Effects of Calcium on Indices of Bone and Cardiovascular Health, and on Cancer

Sarah Marie Bristow

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine, The University of Auckland, 2014.

Abstract

Background

Calcium supplements are used to promote bone health; however, recent evidence suggests they might also increase cardiovascular risk and reduce cancer risk. The relationship between dietary calcium intake and bone health is unclear. This thesis explores the effects of calcium supplements on serum calcium and how these relate to bone and cardiovascular health; relationships between dietary calcium intake and bone health; and relationships between calcium supplementation and cancer risk.

Methods

Four studies were performed.

1. A randomised controlled trial on the acute and 3-month effects of different calcium supplements on serum calcium, bone turnover and indices of cardiovascular disease.
2. A cross-over trial on the acute effects on serum calcium of a calcium supplement with a meal, or calcium from fortified juice or dairy products.
3. An analysis of the relationship between dietary calcium intake and bone mineral density (BMD), change in BMD and fracture risk in postmenopausal women, and whether the effect of calcium supplementation is modified by dietary calcium intake.
4. A meta-analysis of randomised controlled trials on the effect of calcium supplementation on the risk of total, colorectal, breast and prostate cancer.

Results

1. Serum calcium was acutely elevated following a calcium supplement and this was not diminished with continuous use. A supplement which elevated serum calcium less still suppressed bone turnover comparably. Blood pressure and blood coagulability were increased after a calcium supplement compared with a control.

2. A calcium supplement with or without a meal and calcium from fortified juice elevated serum calcium comparably. Calcium from dairy products elevated serum calcium less.
3. Dietary calcium intake was weakly associated with BMD, but not with change in BMD or fracture risk. The effect of calcium supplementation on change in BMD was not modified by dietary calcium intake.
4. Calcium supplementation was not associated with the risk of total cancer.

Conclusions

The acute effects of calcium supplements on serum calcium and indices of cardiovascular disease may explain the increased cardiovascular risk associated with calcium supplementation. Dietary calcium intake was not associated with bone health in postmenopausal women, and calcium supplementation was not associated with short-term cancer risk.

Acknowledgements

Firstly, I would like to extend my deepest gratitude to my primary supervisor Professor Ian Reid, for agreeing to act as my supervisor, and for his guidance, encouragement and support throughout this thesis. I would also like to thank Associate Professor Mark Bolland, who acted as co-supervisor for this thesis, for his ideas and guidance, particularly in the research described in Chapters 6 and 7. Thank you to Greg Gamble, who provided statistical support throughout this thesis. His knowledge and expert advice have been invaluable.

A number of other people have contributed to the research described in this thesis. Angela Stewart contributed a huge amount of time and effort to the trials in Chapters 2 to 4. Her endless support and enthusiasm resulted in these trials running as well as they did. Dr Anne Horne shared her substantial experience in clinical research, and provided guidance throughout both of these trials. Lauren Horne, Dr Rama Kalluru, Meaghan House, Opetaia Aati and Bobby Mihov also assisted with the running of both trials. Chris Finlay provided training and technical support in measuring blood coagulation in Chapter 4. Dr Andreas Pasch, Professor Charles O'Neill and Karen Callon performed the assays of regulators of vascular calcification in Chapter 4. Professor Alison Avenell, Graeme MacLennan and Associate Professor Andrew Grey critically revised the protocol and manuscript of the meta-analyses described in Chapter 7. The original literature search and review of studies for these meta-analyses were performed by Associate Professor Mark Bolland and Associate Professor Andrew Grey.

I would like to extend a huge thank you to the members of the clinical Bone and Joint Research Group I have worked alongside during the past few years: Dr Anne Horne, Angela Stewart, Bobby Mihov, Chris Franklin, Katy Wiessing, Leanne Purvis, Lauren Horne, Meaghan House, Manisha Singh, Opetaia Aati, Paul Tan, Dr Rama Kalluru, Rhonwen Hauraki, Sheryl Fenwick and Zaynah Nisa, for their ongoing support and friendship. Thank you also to all of the women who gave up their time to participate in the studies included in this thesis. Without their enthusiasm for clinical research this work would not have been possible. Finally, I would like to thank my family, for their constant support and encouragement.

Table of Contents

List of Tables

List of Figures

Abbreviations

ANCOVA	Analysis of covariance
AUC	Area under the curve
BMD	Bone mineral density
BMI	Body mass index
CKD	Chronic kidney disease
CTX	C-telopeptide
FAO	Food and Agriculture Organisation of the United Nations
FGF23	Fibroblast growth factor-23
HR	Hazard ratio
IOM	Institute of Medicine
MCH	Microcrystalline hydroxyapatite
PINP	Procollagen type-I N-terminal propeptide
PTH	Parathyroid hormone
RCT	Randomised controlled trial
RECORD	Randomised Evaluation of Calcium or Vitamin D
RR	Relative risk
TEG	Thromboelastography
T_{50}	Transition time of primary to secondary calciprotein particles
WHO	World Health Organisation

THE UNIVERSITY OF AUCKLAND
SCHOOL OF GRADUATE STUDIES

Graduate Centre
ClockTower – East Wing
22 Princes Street, Auckland
Phone: +64 9 373 7599 ext 81321
Fax: +64 9 373 7610
Email: postgraduate@auckland.ac.nz
www.postgrad.auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains research reported in published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. Chapter 3 under review as: Bristow SM, Gamble GD, Stewart A, et al. Acute and 3-month effects of microcrystalline hydroxyapatite, calcium citrate and calcium carbonate on serum calcium and markers of bone turnover: a randomised controlled trial in postmenopausal women. Br J Nutr.	
Nature of contribution by PhD candidate	SMB wrote the protocol, co-ordinated the study, assisted with the analysis of the data, wrote the chapter and manuscript.
Extent of contribution by PhD candidate (%)	80

CO-AUTHORS

Name	Nature of Contribution
Greg Gamble	Analysed the data, critically revised the protocol and manuscript.
A Stewart & L Horne	Assisted with the running of the trial.
O Aaati & M House	Assisted with the running of the trial.
Anne Horne	Assisted with the running of the trial.
Ian Reid	Principal investigator, critically revised the protocol and manuscript.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

Name	Signature	Date
Greg Gamble		9/07/2014
Angela Stewart and Lauren Horne		9/07/2014
Meaghan House		9/07/2014
Opetaia Aati		9/07/2014
Anne Horne		9/07/2014
Ian Reid		9/07/2014

THE UNIVERSITY OF AUCKLAND
SCHOOL OF GRADUATE STUDIES

Graduate Centre
ClockTower – East Wing
22 Princes Street, Auckland
Phone: +64 9 373 7599 ext 81321
Fax: +64 9 373 7610
Email: postgraduate@auckland.ac.nz
www.postgrad.auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains research reported in published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. Chapter 7 has been published as Bristow SM, Bolland MJ, MacLennan GS et al. (2013) Calcium supplements and cancer risk: a meta-analysis of randomised controlled trials. Br J Nutr 110, 1384-1393	
Nature of contribution by PhD candidate	SMB wrote the protocol, updated the literature search, assisted with the analysis of the data, wrote the chapter and the manuscript.
Extent of contribution by PhD candidate (%)	70

CO-AUTHORS

Name	Nature of Contribution
Mark Bolland	Performed the original literature search, critically revised the protocol and manuscript.
Graeme Maclennan	Critically revised the protocol and manuscript.
Alison Avenell	Critically revised the protocol and manuscript.
Greg Gamble	Analysed the data, critically revised the protocol and manuscript.
Ian Reid & Andrew Grey	Critically revised the protocol and manuscript

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

Name	Signature	Date
Mark Bolland	M Bolland	9/07/2014
Graeme MacLennan	Graeme MacLennan	9/07/2014
Alison Avenell	Alison Avenell	9/07/2014
Greg Gamble	Greg Gamble	9/07/2014
Andrew Grey		9/07/2014
Ian Reid	Ian Reid	9/07/2014

CHAPTER 1: INTRODUCTION

Calcium is an essential element in the diet of humans; however, there has long been controversy regarding the optimal intake and the significance of calcium deficiency, particularly in the pathogenesis of osteoporosis. In the 1940s, Albright proposed that while calcium and vitamin D deficiency would result in osteomalacia, postmenopausal osteoporosis was a result of sex hormone deficiency, and unrelated to calcium nutriture [1]. Consistently, it was observed that populations in which calcium intakes were very low did not appear to suffer poorer bone health or greater rates of fracture [2-5]. In 1953, the recommended intake of calcium in the United States and Canada was lowered from 1000 mg/day to 800 mg/day [6, 7]. In a report in 1962, the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO) [8], concluded that "*Most apparently healthy people – throughout the world – develop and live satisfactorily on a dietary intake of calcium which lies between 300 mg and over 1,000 mg a day. There is so far no convincing evidence that, in the absence of nutritional disorders and especially when the vitamin D status is adequate, an intake of calcium even below 300 mg or above 1,000 mg a day is harmful.*" Based on this report, in 1974, the FAO and WHO recommended even lower intakes of calcium for adults of 400 – 500 mg/day [9].

In the decades following this report, opinions shifted, and an inadequate intake of calcium became widely regarded to play some, and possibly an important, role in the pathogenesis of osteoporosis. During these decades a large body of research on the relationship between calcium nutrition and bone health was performed. An influential series of calcium balance studies indicated that intakes below 1000 - 1500 mg/day were inadequate to replace obligatory calcium losses in women [10, 11]. In one of the earliest large trials of calcium supplements, substantial reductions in bone loss and fracture risk were observed with calcium and vitamin D supplementation in an elderly population [12]. Far more modest, although still beneficial, effects were reported in other trials of calcium supplements [13-16]. Correspondingly, in the United States and Canada, the recommended adequate intake of calcium for adults aged over 50 years was 1200 mg/day in 1997 [17], and the FAO and WHO recommended intake of calcium for postmenopausal women and men over 65 years was 1300 mg/day in 2002 [18]. Similarly, in Australia and New Zealand, the recommended intake of calcium for postmenopausal women and older men was 1300 mg/day in 2006 [19]. However,

there still existed some disagreement regarding the optimal intake of calcium, as the recommended intake of calcium in the United Kingdom remained at 700 mg/day for all adults [20, 21].

These high recommended intakes of calcium are clearly difficult for most people to achieve. In New Zealand, the calcium intakes of men and women aged 50 – 70 years are 830 and 740 mg/day, and in those aged over 70 years, 740 and 670 mg/day [22]. In Australia, calcium intakes in men and women aged over 70 years are 780 and 680 mg/day [23]. Ninety percent of men and 95% of women in this age group do not meet the recommended levels. In the United States, dietary calcium intakes among men and women aged 50 – 70 years are 950 and 790 mg/day. Twenty-two percent of men and 9% of women in this age group have calcium intakes above the recommended adequate intake [24]. Among men and women aged over 70 years, intakes are 870 and 750 mg/day, with 15% and 8% above the recommended adequate intake. In response to the high prevalence of "dietary calcium deficiency" and its purported role in osteoporosis, calcium supplementation of the diet has become widespread. In the United States, 65% of women and 51% of men aged over 50 years report using calcium supplements, which contribute 600 and 300 mg/day to total calcium intake, respectively [24]. In a sample of adults aged over 65 years in Australia, 82% of women and 18% of men reported using calcium supplements [25]. A further response to this "dietary calcium deficiency" is the increased fortification of a number of different foods with additional calcium [26, 27].

Against this background of widespread calcium supplementation, the Institute of Medicine (IOM), in their most recent report on calcium and vitamin D requirements in 2011 [28], took a more cautious approach, stating "*There is controversy concerning levels of nutrient intake, and at times the concept that "more is better" emerges. However, for both calcium and vitamin D, there is another underlying question: How much is too much?"* In light of an apparent increase in kidney stone risk with calcium supplementation, the IOM set tolerable upper limits for calcium of 2,000 mg/day. However, their recommended intakes did not change, remaining at 1,200 mg/day in postmenopausal women and older men. Around this time, evidence also emerged from a meta-analysis of randomised controlled trials (RCT) suggesting that calcium supplementation increases the risk of myocardial infarction and stroke [29]. Furthermore, calcium with vitamin D supplementation was suggested to lower cancer risk [30]. Thus, in recent years, it has become increasingly evident that the non-skeletal effects of calcium supplementation must be considered when evaluating the role of

calcium supplements in bone health. The increased risk of kidney stones and cardiovascular events associated with calcium supplementation have resulted in recent statements that, for bone health, the recommended intakes of calcium should be met through diet alone [31, 32].

Interestingly, there is evidence to suggest that calcium from supplements, i.e. that supplied as a large concentrated bolus of an isolated nutrient, might have a different effect on several health outcomes from that of calcium supplied through the diet. It is already accepted that calcium supplementation increases kidney stone risk, while the same increase in risk does not apply to high dietary calcium intakes [33-35]. High dietary calcium intakes similarly do not appear to be associated with the same increase in cardiovascular risk as calcium supplements [36]. Furthermore, while calcium supplements modestly reduce the risk of fracture [37], there is less evidence of a relationship between dietary calcium intake and fracture [38-40]. It is unclear why supplemental and dietary calcium might have different effects on bone and cardiovascular health. A possible reason could be the increase in serum calcium that occurs following the ingestion of a calcium supplement, as this might be smaller after the ingestion of calcium from the diet.

An improved understanding of calcium nutrition is presently of key importance because of the widespread use of calcium supplements; the large variations in dietary calcium intake that exist both between and within populations; and the known or suspected effects of calcium on osteoporosis, cardiovascular disease and cancer, diseases which contribute substantially to morbidity and mortality worldwide. The aim of this thesis is to explore the acute effects of different calcium sources on serum calcium and how these relate to bone and cardiovascular health, the relationship between dietary calcium intake and bone health, and the relationship between calcium supplementation and cancer risk.

Specifically, in Chapter 3 I will present the results of an RCT of postmenopausal women which examined the acute and 3-month effects of different calcium supplements on serum calcium and bone turnover, and whether any acute effects on serum calcium were diminished over time. In Chapter 4 I will present further findings from this RCT on the acute and 3-months effects of calcium supplements on indices of cardiovascular disease including blood pressure, blood coagulation and regulators of vascular calcification. In Chapter 5 I will present the findings from a randomised, cross-over trial of postmenopausal women which compared the acute effects on serum calcium of a calcium supplement, a calcium supplement taken after a meal, calcium from fortified juice and calcium from dairy products. In Chapter 6

I will present the results from an analysis on the relationship between dietary calcium intake and bone mineral density (BMD), change in BMD and fracture risk in postmenopausal women, and whether the effects of calcium supplements on change in BMD and fracture risk are related to dietary calcium intake. Finally, in Chapter 7 I present the results of trial and patient level meta-analyses of RCTs on the relationship between calcium supplementation and the risk of total, colorectal, breast and prostate cancer.

CHAPTER 2: LITERATURE REVIEW

The literature review is divided into four sections. The first section will review calcium metabolism; the second section dietary calcium intake, calcium supplementation and bone health; the third section dietary calcium intake, calcium supplementation and cardiovascular health; and the fourth section calcium supplementation and cancer risk.

2.1 CALCIUM METABOLISM

This section will describe the distribution of calcium in the body, how the extracellular calcium concentration is maintained by parathyroid hormone (PTH), vitamin D and calcitonin, and the balance between calcium intake and output.

Calcium distribution

The adult body contains around 1000 g of calcium. Of this, 99% is located in the mineral phase of bone as crystalline hydroxyapatite. The function of calcium in bone is to provide mechanical strength to the surrounding collagenous tissue, and a ready source of ions for mineral homeostasis. The remaining 1% of calcium is located in the extracellular and intracellular fluid of blood and soft tissues. The concentration of calcium in the extracellular fluid is 10^{-3} M. In serum, 45% of calcium is protein bound, mainly to albumin, 10% is complexed with small anions such as citrate and phosphate, and 45% is in the free, ionised state. Only the ionised portion of calcium is able to move in and out of cells and participate in cellular processes. Calcium bound to albumin is biologically inert, but provides a readily available source of calcium when ionised calcium concentrations are low. The concentration of calcium in normal serum ranges from around 2.1 to 2.5 mmol/l, and the concentration of ionised calcium from 1.15 to 1.30 mmol/l. The concentration of calcium in the intracellular fluid is 10^{-6} M, creating a 1000-fold difference between extracellular and intracellular calcium, favouring calcium entry into cells. Pulsatile peaks in intracellular calcium, where the concentration may be increased 10 to 100-fold, occur through ligand-induced activation of

cell surface receptors. Peaks in intracellular calcium act as a key second messenger for a diverse range of processes including cell proliferation, differentiation and apoptosis; hormone secretion, muscle contraction and nerve transmission.

Calcium homeostasis

Due to the importance of calcium for normal cell functioning, the extracellular calcium concentration is tightly regulated within a narrow range. This range is under the control of an endocrine feedback system involving most notably the calcitropic hormones PTH, vitamin D and calcitonin. This system attempts to maintain ionised calcium concentrations to within ~10% by regulating the amount of calcium absorbed in the gut, excreted through the kidneys and released from and accreted into bone.

Parathyroid hormone

PTH is expressed in humans exclusively in the parathyroid gland, and is secreted in response to minor decrements in ionised calcium which are detected through the calcium-sensing receptor expressed on chief cells in the parathyroid gland. The role of PTH is to restore ionised calcium concentrations to normal. A rise in extracellular calcium results in the suppression of PTH synthesis and secretion. When extracellular calcium concentrations fall, the suppression of PTH synthesis and secretion is lifted. Changes in extracellular calcium can have immediate and long-term effects on PTH secretion. Short-term increases in extracellular calcium result in increased cleavage of PTH and inhibition of PTH secretion, while long-term changes alter the expression of the PTH gene [41] and the number of parathyroid cells which secrete PTH [42]. PTH secretion is also regulated by 1,25-dihydroxyvitamin D and phosphate. PTH increases the activity of the enzyme which converts 25-hydroxyvitamin D to its more active form, 1,25-dihydroxyvitamin D, which then acts in a negative feedback manner to inhibit PTH secretion [43]. Increased phosphate increases PTH secretion, an effect due in part to the complexing of phosphate with ionised calcium, and in part to a direct effect of phosphate on the parathyroid gland [44].

The role of PTH is to increase extracellular calcium concentrations through its effects on bone and the kidneys, and through an indirect effect on the gut. In bone, PTH acts on osteoblast lineage cells to increase the expression of receptor activator of nuclear factor κ β ligand (RANKL) which enhances osteoclastogenesis and osteoclast resorbing activity releasing calcium and phosphate into the serum. In the kidney, PTH increases the reabsorption of calcium and decreases the reabsorption of phosphate. As both calcium and phosphate are released during bone resorption, and as phosphate complexes with ionised calcium, by decreasing the renal reabsorption of phosphate PTH permits a rise in both total *and* ionised calcium. In the kidney, PTH also increases the rate at which 25-hydroxyvitamin D is converted to its active form, 1,25-dihydroxyvitamin D, by activating the 1α-hydroxylase enzyme. PTH thereby indirectly enhances calcium absorption in the gut. These actions of PTH increase extracellular calcium and 1,25-dihydroxyvitamin concentrations, which in turn act in a negative feedback manner to decrease PTH secretion.

Vitamin D

Vitamin D is a major regulator of calcium and phosphate homeostasis which acts with PTH to increase extracellular calcium. Vitamin D is formed through sunlight-dependent endogenous production in the skin. Vitamin D can also be obtained from some foods in the diet; however in most areas of the world the contribution of diet to vitamin D status is minimal. Endogenous vitamin D production involves the photolysis of the precursor 7-dehydrocholesterol in the skin to previtamin D by UV-B radiation. Previtamin D then undergoes a temperature-dependent isomerisation to vitamin D. Vitamin D binds to vitamin D binding protein, and is transported to the liver, where it is hydroxylated to 25-hydroxyvitamin D. The final step in its activation is a further hydroxylation in the kidneys to 1,25-dihydroxyvitamin D by the renal 1α-hydroxylase enzyme. The primary regulator of this enzyme is PTH. Thus, a decrease in the extracellular calcium concentration results in a rise in PTH and thereby a rise in 1,25-dihydroxyvitamin D.

The traditional role of vitamin D is to increase calcium and phosphate absorption in the gut. Calcium is absorbed via two pathways: an active, transcellular pathway that is totally dependent on 1,25-dihydroxyvitamin D, and a passive, paracellular route that is driven by the luminal calcium concentration. At low and medium calcium intakes, 1,25-dihydroxyvitamin

D-mediated active calcium absorption is the predominant route of absorption. At higher calcium intakes, 1,25-dihydroxyvitamin D is suppressed, and the proportion absorbed by passive diffusion increases. Fractional calcium absorption thus varies inversely with calcium intake [45, 46]. In bone, like PTH, 1,25-dihydroxyvitamin D acts on osteoblasts to increase production of RANKL [47], resulting in increased osteoclastic bone resorption and the release of calcium and phosphate into serum. Increased 1,25-dihydroxyvitamin D and increased extracellular calcium inhibit PTH, thereby inhibiting further production of 1,25-dihydroxyvitamin D.

Calcitonin

Calcitonin is secreted from the C-cells in the thyroid gland. The primary role of calcitonin is to decrease serum calcium and phosphate to below a certain set-point and it therefore acts as a physiologic antagonist of PTH. Calcitonin is secreted from the thyroid gland in response to small increments in ionised calcium detected by the calcium-sensing receptor, with the rate of calcitonin secretion determined by the extracellular concentration. The main target organs of calcitonin are bone and the kidney. In bone, calcitonin suppresses osteoclastic bone resorption, decreasing the amount of calcium and phosphate released into serum. In the kidney, calcitonin decreases the renal reabsorption of calcium and phosphate, increasing their excretion. As specific symptoms of calcitonin excess and deficiency in relation to calcium and bone homeostasis have not been identified, the exact role of calcitonin is not clear.

Calcium balance

Calcium balance describes the balance between calcium intake through the diet and calcium output in the faeces and urine, and to a lesser degree, through the skin. Calcium balance may be positive, where intake exceeds output; neutral, where intake is equal to output; or negative, where intake is less than output. Calcium balance will vary according to life stage: in childhood and adolescence, balance will be positive, indicating bone gain; in adults balance will be neutral, indicating bone maintenance; and in older adults balance will tend to be slightly negative, indicating age- and menopause-related bone loss. The appropriate calcium

balance is maintained through the actions of PTH, vitamin D and calcitonin, which respond to changes in calcium intake and the resulting changes in extracellular calcium by altering calcium output. However, calcium output cannot be reduced to zero, due to the obligatory losses of calcium in the urine (around 150 mg/day) and skin (40 mg/day). It is therefore assumed that a negative calcium balance will occur when the amount of calcium absorbed in the gut is less than ~200 mg/day [48]. When calcium balance is negative, extracellular calcium levels can only be maintained at the expense of the loss of calcium from bone. Much attention has therefore focused on determining the level of calcium intake that will result in an adequate amount of calcium being absorbed. Complicating this relationship are a number of factors known or suspected to influence calcium absorption and/or output such as age, 25-hydroxyvitamin D status, and intakes of protein and sodium [45, 49, 50]. Therefore, there has been ongoing disagreement regarding the calcium intake required for neutral calcium and bone balance.

Studies of calcium balance have been used to determine the calcium intake required for neutral calcium balance. These studies been carried out in different age groups and populations, and under different dietary conditions, and have had inconsistent findings [5, 10, 11, 51-57]. The most influential calcium balance studies to-date are a series by Heaney and colleagues in the 1970s. In their first report, Heaney et al described calcium balance in 130 perimenopausal nuns aged 35 to 50 years [11]. Calcium balance in the total group was slightly negative, averaging a loss of – 24 mg/day. A positive correlation between calcium intake and calcium balance was reported, with women on higher intakes exhibiting a more positive (or less negative) calcium balance. The calcium intake predicted from their data to produce a neutral calcium balance was 1240 mg/day. In a second report in this group of women (n = 168), calcium balance was examined by menopausal status [10]. In premenopausal women and menopausal women treated with estrogen, calcium balance averaged – 19 mg/day. In postmenopausal women not treated with estrogen, calcium balance averaged – 43mg/day. However, as the postmenopausal women in this study were in early menopause (<5 y post-menopause), a time of rapid bone loss due to estrogen withdrawal, the degree of negative calcium balance will be greater than that present in women >5 years post-menopause. Calcium balance in this study was again reported to be positively correlated with calcium intake. The calcium intake predicted from their data to produce a neutral calcium balance was 990 mg/day for premenopausal and estrogen-treated women, and 1504 mg/day for postmenopausal women.

More recently, Hunt and Johnson examined data from a series of 19 balance studies, which together included 73 women aged 20 to 75 years (mean 47 years) and 82 men aged 19 to 64 years (mean 28 years) [58]. The calcium intake predicted from their data to produce a neutral calcium balance was 741 mg/day, regardless of age or sex. As around half of the women were over 50 years, this value is very different to the 1500 mg/day suggested for postmenopausal women in the earlier balance studies [10]. Hunt and Johnson also noted that calcium balance appeared to be tightly controlled over the range of calcium intakes studied, from 400 to 1700 mg/day.

The findings of calcium balance studies have been influential in developing recommended intakes of calcium [28]. However, their precision in estimating the relationship between calcium intake and bone balance is unclear. In the early balance studies, calcium intake was correlated with calcium balance, which may have resulted in a spurious relationship, as intake is used in the calculation of balance. Calcium balance studies are difficult to perform and involve a high degree of participant cooperation, including living in a controlled metabolic unit. Very long-term studies of calcium balance are therefore not feasible. The accuracy of calcium balance studies depends on whether individuals are consuming their usual calcium intakes, as an increase or decrease in intake requires a period of adaption before homeostasis is restored. The IOM has recommended that calcium balance studies be performed in individuals consuming their usual self-selected calcium intakes, and if this is not possible, then a period of adaption to the altered intake should be allowed for [28]. A 7-day period of adaption was suggested as adequate. However, fractional calcium retention and 1,25-dihydroxyvitamin D were different 1 week after a change in calcium intake compared to 8 weeks [59], and calcium absorption changed for at least 8 weeks after calcium intake was altered [60]. One study reported that 9 days on a high-calcium diet resulted in a positive calcium balance of +460 mg/day, which is presumably not maintained long-term [51]. Bone turnover might require a period of months to reach a new steady state when calcium intake is altered [61, 62]. Thus, it is possible that differences in short-term calcium balance might not translate into the expected differences in long-term bone balance.

Summary of calcium metabolism

Calcium is essential for a diverse range of cellular processes and provides structural integrity and mechanical resistance to bone. Extracellular calcium levels are maintained through the concerted actions of PTH, vitamin D and calcitonin. When extracellular calcium is low, concentrations are increased to normal by increased calcium reabsorption in the kidneys, increased calcium absorption in the gut and increased osteoclastic bone resorption. When extracellular calcium is increased, concentrations are decreased to normal by decreased calcium reabsorption in the kidneys, decreased calcium absorption in the gut, and decreased osteoclastic bone resorption. Calcium balance refers to the balance between calcium intake and output. In normal adults, calcium balance should be neutral or slightly negative, reflecting bone maintenance or age- and menopause-related bone loss. However, as there is always an obligatory loss of calcium in the urine and from the skin, there must be a threshold of calcium intake below which calcium balance becomes increasingly negative. What this calcium intake is has been the subject of debate. Early calcium balance studies suggested intakes of 1500 mg/day were required for neutral calcium balance in postmenopausal women, while more recently; 740 mg/day was suggested as adequate. The precision of such studies in estimating the long-term relationship between calcium intake and bone health is unclear.

2.2 CALCIUM AND BONE HEALTH

Calcium has a key structural role in bone and bone participates in calcium homeostasis. As such, calcium is the nutrient most universally associated with bone health. The calcium intakes of populations often fall below the recommended levels [22, 63, 64], and increasing calcium intake, whether through diet or supplements, has been widely encouraged to improve bone health [65-67]. Bone is in reality, however, a collagen-based connective tissue and increasing calcium intake does not simply result in more calcium adhering to the skeleton. Changes in bone mass can only be mediated through a change in the balance of osteoclastic bone resorption to osteoblastic bone formation. As I described earlier, a reduction in calcium intake which reduced extracellular calcium should result in increased PTH and increased osteoclastic bone resorption. Conversely, an increase in calcium intake which increased extracellular calcium should result in decreased PTH and lowered osteoclastic bone

resorption. This effect has been clearly demonstrated acutely following the ingestion of a calcium supplement [68-71], and appears to translate into a small reduction in bone loss, and possibly fracture risk, in clinical trials of calcium supplements [37].

What is less clear is whether long-term dietary calcium intakes are associated with the same effects on bone as supplements. Within populations, a null relationship between dietary calcium intake and fracture risk is often reported [72], and between populations, fracture risk is not increased in those where low calcium intakes are the norm, such as Asia and regions of Africa [73, 74]. There is thus uncertainty as to whether meeting the recommended dietary intakes of calcium will actually benefit bone health, and this is reflected in the absence of calcium intake from fracture risk calculators in routine clinical use (FRAX and the Garvan Fracture Risk Calculator [75, 76]). The recommended intakes of calcium have been based heavily on the findings of calcium balance studies [28], which prior to the advent of bone densitometry, were relied on to assess the relationship between calcium intake and bone balance. Bone densitometry is now available to provide an accurate measure bone balance over long periods of time in cohorts consuming their usual diets. This section will review the evidence relating to dietary and supplemental calcium intake and bone mass and fracture risk, and discuss how calcium intake might influence these outcomes.

Dietary calcium intake and bone health

The relationship between dietary calcium intake, BMD and fracture risk has been widely studied; however the evidence is primarily observational. There is therefore the risk of confounding, as high dietary calcium intakes are associated with several factors which might influence bone health including age, calcium supplement use, smoking status, alcohol consumption, physical activity and socioeconomic status [36, 77-79]. High calcium intakes are usually reflective of high dairy intakes, and high dairy intakes are associated with higher intakes of a range of nutrients, including protein and vitamin D [80-82]. Calcium intake will also be positively correlated with energy intake and will tend to be higher in larger or more active individuals. Alternatively, those who have been diagnosed as at risk of osteoporosis might have increased their calcium intake, resulting in an inverse association between calcium intake and BMD or fracture risk.

Bone mineral density

The relationship between calcium intake and BMD has been examined in prospective cohort and cross-sectional studies. Some small intervention studies have also reported changes in BMD following a dietary calcium intervention, usually calcium-fortified milk.

Longitudinal studies

Most prospective cohort studies have reported no association between dietary calcium intake and bone loss. In a study of 106 women, Riggs reported that calcium intake (range 260 – 2035 mg/day) was not associated with change in BMD over 4 years at the midradius or lumbar spine [83]. In a study of 154 perimenopausal women, van Beresteijn reported that cortical radius BMC loss over 8 years was not different across tertiles of dietary calcium intake ranging from less than 800 mg/day to more than 1350 mg/day [84]. In a study of 398 early postmenopausal women, Hosking et al reported that total and dietary calcium intakes (1055 and 876 mg/day, respectively) were not associated with bone loss over 2 years at the lumbar spine, total hip, total body or forearm [85]. In a study of 173 men and 143 women, Dennison et al reported that total calcium intake (mean 719 mg/day in men and 642 mg/day in women) was not associated with bone loss at the lumbar spine, femoral neck or total femur over 4 years [86]. In a study of 800 older men and women, Hannan et al reported that total calcium intake (mean 810 mg/day in men and 783 mg/day in women) was not associated with bone loss over 4 years at the femoral neck, trochanter, radial shaft, lumbar spine, Ward's area or ultradistal radius [87]. In a study of 122 postmenopausal women, Reid et al reported that dietary calcium intake (mean 762 mg/day) was not associated with bone loss over 2 years at the total body, lumbar spine or hip [88]. In a study of 133 premenopausal and 134 postmenopausal women with high or low physical activity levels and high (more than 1200 mg/day) or low (less than 800 mg/day) calcium intakes, Uusi-Rasi et al reported calcium intake was not related to femoral neck or trochanter BMC loss over 10 years [89]. In a study of 121 early postmenopausal women, Hansen et al reported dietary calcium intake was not related to BMC loss at the forearm over 12 years [90]. In a study of 774 elderly Japanese women, Nakamura et al reported that forearm bone loss was not different across quartiles of dietary calcium intake ranging from less than 433 mg/day to more than 766 mg/day [91].

Other studies have reported associations between calcium intake and bone loss at certain sites, in subgroups of participants or among those with very low calcium intakes. In a study of 891 perimenopausal women, Macdonald et al reported that dietary calcium intake (mean 1055 mg/day) was correlated with change in femoral neck BMD over 6 years (r = 0.07) but not with change in lumbar spine BMD [92]. In a study of 454 postmenopausal Chinese women, bone loss over 18 months was less for those with dietary calcium intakes of more than 934 mg/day compared with those with intakes of less than 341 mg/day at the total body (-1.54% versus -0.97%) and Ward's area (-1.49% versus – 0.28%), but not different at the lumbar spine, total hip, femoral neck, trochanter or intertrochanter [93]. In a study of 1,856 men and 2,452 women, Burger et al reported a trend for the annual rate of femoral neck bone loss to decrease across quartiles of dietary calcium intake (ranging from less than 900 mg/day to more than 1300 mg/day) in men, whereas no such no such relationship was observed in women [94]. In a study of 954 perimenopausal women, Sirola et al examined bone loss by smoking status and tertiles of dietary calcium ranging from less than 650 mg/day to more than 930 mg/day. Among nonsmokers, the annual rate of bone loss at the lumbar spine was lower in the second and third tertiles compared with the first tertile (-0.41% and -0.35% versus -0.61%), and femoral neck bone loss lower in the third tertile compared with the first tertile (-0.55% versus -0.72%) [95]. Among ever smokers, there was no relationship between dietary calcium intake and bone loss. In a study of 76 postmenopausal women, Dawson-Hughes examined bone loss at the spine over 7 months by quartiles of dietary calcium ranging from less than 405 mg/day to more than 777 mg/day [96]. The annual rate of bone loss at the spine across the quartiles (first to fourth) was -4.8%, +1.1%, -2.3% and +2.5%, with the difference between the first and fourth quartiles significant.

Cross-sectional studies

A large number of cross-sectional studies have examined the relationship between dietary calcium intake and BMD, and only larger studies or those performed in non-white populations will be reviewed here. Most studies have reported an association between calcium intake and BMD at least at one body site. In a study of 4,434 women, Aptel et al reported that a 100 mg/day increase in dietary calcium was associated with 0.2% higher femoral neck BMD, and a 100 mg/day increase in calcium from drinking water associated with a 0.5% higher femoral neck BMD [97]. In a study of 5,995 men, Cauley et al reported

that a 390 mg/day increase in dietary calcium intake was associated with a 0.6% higher femoral neck BMD and with a 0.4% higher lumbar spine BMD [98]. In a study of 9,704 women, Bauer et al reported that a 400 mg/day increase in dietary calcium intake was associated with a 0.7% higher distal radius bone mass [99]. Among 1,600 women, Kroger et al reported that calcium intake was not associated with femoral neck or lumbar spine BMD in multiple regression analysis; however women with a calcium intake of less than 520 mg/day had a 60% increased risk of a low BMD (< -1 SD) at the femoral neck but not at the lumbar spine [100].

A stronger relationship between calcium intake and BMD might exist in men. In a study of 554 women and 191 men, McCabe reported that, among men, dairy calcium and dietary calcium, but not non-dairy calcium, were positively correlated with total hip and femoral neck BMD (r = 0.21 – 23), but among women, there was no association [101]. In a study of 1,075 women and 690 men, Nguyen et al reported that women in the highest tertile of dietary calcium intake (more than 710 mg/day) had a 2.5% higher BMD than those in the lowest tertile (less than 460 mg/day), but no difference in lumbar spine BMD [102]. The relationship was more pronounced in men: among those in the highest tertile of dietary calcium intake, femoral neck and lumbar spine BMD were 5% higher than those in the lowest tertile. Nguyen et al also reported a significant interaction with body mass index (BMI), such that the relationship between dietary calcium and BMD was only present in women with a BMI of less than 23 kg/m^2 and in men less than 27 kg/m^2. Andersen et al examined the relationship between total calcium intake and hip and spine BMD in 1,354 men and women [79]. Among those aged 50 to 70 years, total calcium intake was not associated with femoral neck BMD. Among men aged 71 years or more, femoral BMD was significantly higher in the fifth quintile of total calcium intake compared with the fourth and first quintiles (0.79 g/cm^2 versus 0.71 g/cm^2 and 0.72 g/cm^2). Among women aged 71 years or more, femoral neck BMD was significantly lower in fifth quintile of total calcium intake compared with the second quintile (0.62 g/cm^2 versus 0.68 g/cm^2). Total calcium intake was not associated with lumbar spine in either age group or sex.

In addition to the study by Nguyen [102], two further studies have suggested that the relationship between calcium intake and BMD is dependent on weight status. In contrast to the findings of Nguyen, Korpelainen reported that among 1,222 older women, a lifetime low dietary calcium intake (less than 654 mg/day) increased the risk of a low ultradistal radius BMD only among women with a BMI between 25.1 and 28.5 kg/m^2 [103]. In a study of

1,771 women within 5 years of menopause, Varenna et al reported that dairy intake was inversely associated with overweight status, and that the negative effect of a low dairy (or low calcium) intake on BMD was attenuated by a higher BMI [104].

The relationship between calcium intake and BMD could also depend on physical activity status. In a study of 1,254 older women, Mavroeidi reported no association between tertile of total calcium intake and lumbar spine or total hip BMD, but a significant interaction between calcium intake and physical activity [105]. Among women with a low (mean 942 mg/day) or medium (1,042 mg/day) calcium intake, BMD tended to increase across tertiles of physical activity, whereas in women with a high calcium intake (1,391 mg/day) no relationship between physical activity and BMD was observed. In a study of 1,343 older women, Devine et al reported that after adjusting for physical activity and other covariates, a medium or high calcium intake (more than 1050 mg/day) compared with a low intake (less than 790 mg/day) was associated with a 1.8% higher total hip BMD, but no difference in femoral neck or intertrochanter BMD [106]. Among participants in the highest tertile of calcium intake and physical activity, total hip BMD was 5.1% higher than those in the lowest tertile of calcium and physical activity.

Alternatively, a relationship between calcium intake and BMD might depend on vitamin D status. In a study of 4,958 women and 5,003 men, Bischoff-Ferrari et al reported that among women who were vitamin D deficient (25-hydroxyvitamin D less than 50 nmol/l), a 300 mg/day increase in dietary calcium intake was associated with a 0.03 g/cm^2 higher BMD at the hip, whereas there was no relationship among those who were vitamin D replete [107]. In men, there was no relationship between dietary calcium intake and BMD at any vitamin D status.

Several cross-sectional studies have been performed in Asian populations. Hu et al examined dietary calcium intakes in 843 Chinese women from five rural counties [82]. Dairy, calcium and protein intakes and BMD at the radius were significantly higher in the one pastoral county compared with the other counties. In the total cohort, BMD was positively correlated with total calcium intake (r = 0.27-0.38) and dairy calcium intake (r = 0.34-0.40), and to a lesser degree with nondairy calcium intake (r = 0.06-0.12). Yano et al studied 1,208 Japanese men and 916 women aged living in Hawaii [108]. In men, dietary calcium intake (mean 509 mg/day) was positively associated with BMC at the distal radius, distal ulna and heel (r = 0.06 - 0.08). In women, dietary calcium intake (mean 434 mg/day) was not associated with

BMC at any site. In a study of 129 rural Thai women with very low calcium intakes (mean 236 mg/day), Pongchaiyakul et al reported that a 1000 mg/day increase in calcium intake was associated with 0.01 g/cm^2 higher distal radius BMD and a 0.04 g/cm^2 higher femoral neck BMD [109]. Dietary calcium was not significantly associated with lumbar spine BMD. The risk of osteoporosis was 40% greater in those with dietary calcium intakes less than 138 mg/day compared with higher intakes. Similar findings were reported in a second study in rural Thai women by the same investigators [110]. In a study of 1,654 Chinese women, Khoo et al reported that a 20 mg/day increase dietary calcium intake was associated with a 0.08% higher BMD at the total hip and a 0.1% higher BMD at the spine [111]. In a study of 407 Chinese men, Cheung et al reported that calcium intake was positively correlated with hip but not spine BMD, however the association did not persist after including age and weight in the model [112]. Similarly, in a study of 2,000 Chinese men, Lau et al reported a weak association between calcium intake and hip and lumbar spine BMD, which diminished after adjustment for weight [113].

Two large studies have assessed the odds of osteoporosis (BMD T-score -2.5 or less) by calcium intake. Among 76,507 postmenopausal women participating in the National Osteoporosis Risk Assessment Study, lifetime dietary calcium intake was calculated from the frequency of milk, cheese, yoghurt and ice-cream consumption. The calculation of current total calcium intake included calcium from supplements and fortified fruit juice. After adjusting for multiple variables including age and BMI, the odds of osteoporosis were 25% lower in those with current total calcium intakes of 800 mg/day or more compared with less than 500 mg/day, and the corresponding risk for lifetime dietary calcium intake was 20% lower [114]. In the Swedish Mammography Cohort, BMD was measured in a subgroup of 5,022 women [77]. After adjusting for multiple covariates including age, BMI and energy intake, the odds of osteoporosis were increased by 47% in the first quintile of dietary calcium intake (less than 751 mg/day) compared with the third quintile (882 to 996 mg/day), but not further decreased in the fourth and fifth quintiles.

Intervention studies

Few trials have assessed the effect of interventions with dietary calcium sources on BMD, and those that have are small in size. These studies are challenging to perform and interpret, as the intervention may provide additional nutrients and energy, and participants cannot

usually be blinded. Most of these studies have used foods fortified with additional calcium, perhaps illustrating the difficulty in increasing dietary calcium intake to a large degree through unfortified foods.

Most trials have assessed the effects on BMD of an intervention with milk fortified with additional calcium. Daly et al randomised 167 men to calcium- and vitamin D-fortified milk, providing an additional 1000 mg/day of calcium, or to a control group who received no intervention [115]. After 2 years, the percentage change in BMD was 0.9 – 1.6% less at the femoral neck, total hip and ultradistal radius in the milk group compared with the control group. Body weight remained unchanged. Nelson et al randomised 36 active and non-active postmenopausal women participating in an exercise intervention trial to a high-calcium milk providing 831 mg/day of calcium or a placebo drink [116]. After 12 months femoral neck BMD increased by 2.2% in the milk supplement group and decreased by 1.1% in the control group, but was not different between the groups at the lumbar spine or distal radius. Kukuljan et al randomised 180 men to exercise, a calcium- and vitamin D-fortified milk providing 1000 mg/day of calcium, both exercise and milk, or no intervention [117]. After 12 months, lumbar spine BMD increased in all groups by 1.4 – 1.5% relative to the control group, but did not change at the hip in the milk group relative to the control.

Several studies have examined the effects of calcium-fortified milk products on bone health in Asian populations. Chee et al randomised 200 postmenopausal Chinese women to a calcium-fortified milk powder providing 1200 mg/day of calcium or to a control group who received no intervention. After 2 years, bone loss was significantly lower in the milk group compared with control group at the total body (-0.13% versus -1.04%), lumbar spine (-0.13 versus -0.90%), femoral neck (-0.51% versus -1.21%) and total hip (0.51% versus -2.17%) [118]. Body weight did not change. Similarly, Lau et al randomised 200 postmenopausal Chinese women to a calcium-fortified milk powder providing 800 mg/day of calcium or a control group who received no intervention [119]. After 24 months, the mean percentage change in BMD in the milk group compared with the control group was significantly lower at the total hip (-0.06% versus -0.88%), lumbar spine (-0.56% versus -1.5%) and total body (-0.32% versus -1.2%). The milk group gained more weight than the control group over the study; however the effects remained significant after changes in body weight were adjusted for. It is possible that milk might have beneficial effects on bone independent of its calcium content. This was suggested in a study by Gui et al in 141 Chinese women randomised to 250 ml/day of milk or a calcium-fortified soy milk, each providing an additional 250 mg/day of

calcium, or a control group who received no intervention [120]. After 18 months, BMD tended to decline or not change at the spine, femoral neck and total hip in the calcium-fortified soy milk and control groups, whereas in the milk group, BMD significantly increased by 2.5% at the hip and 2.8% at the femoral neck. Changes in weight were not reported.

Four studies appear to have used interventions with unfortified milk or milk powder (fortification of the food was not described in their methods). Recker and Heaney randomised 22 postmenopausal women to 800 ml/day of milk or a control group who received no intervention [121]. Calcium intake increased by 800 mg/day in the milk group. After 2 years, BMC had not changed in either group, although bone turnover was significantly lower in the milk group compared with the control group. Baran et al randomised 37 women to increased dairy consumption or usual diet for 3 years. Women in the dairy group increased their calcium intake by an average of 610 mg/day (of note, protein intake also increased by 40 g/day, fat intake by 5 g/day and energy intake by 470 kcal/day in the dairy group but not the control group) [122]. Weight increased by 4.2 kg in the dairy group and 3.4 kg in the control group. Vertebral BMD did not change in the dairy group over 3 years, but significantly decreased by -2.9% in the control group. Prince et al randomised 168 women to 1000 mg/day of calcium carbonate, 84 g/day of milk powder or a placebo [123]. Calcium intake increased by 1000 mg/day in the milk powder group. After 2 years, bone loss in the milk powder and calcium carbonate groups was significantly lower than the control group at the at the intertrochanter (milk +0.07%, carbonate +0.17% and control -0.81%), femoral neck (milk -0.18%, carbonate -0.18% and control -0.67%) and ultradistal tibia (milk -1.5%, carbonate -1.6% and control -2.5%). Storm et al randomised 60 older postmenopausal women to four glasses of milk per day, 1000 mg/day of calcium carbonate or a placebo. Calcium intake increased by 1000 mg/day in the supplement group and by only 400 mg/day in the milk group. After 2 years, bone loss at the greater trochanter was prevented in the carbonate group and was less in the milk group compared with the control group (milk -1.5% and control -3.0%). Changes in lumbar spine BMD (carbonate +3.7%, milk and control no change) and femoral neck BMD (carbonate +3.0%, milk -1.8% and control -0.3%) were not different between the milk and control groups.

Fractures

Ultimately, if higher intakes of dietary calcium have a protective effect on bone mass, then this should translate into a reduction in fracture risk. All studies examining the relationship between dietary calcium intake and fracture risk are observational. No intervention studies with dietary calcium have been large or long enough to examine fracture outcomes.

Most studies have found no association between dietary or total calcium intake and fracture risk. Wickham et al followed 720 men and 690 women for a period of 15 years during which 44 hip fractures occurred [124]. Dietary calcium intake (mean 870 and 730 mg/day in men and women) was not associated with hip fracture risk. Paganini-Hill followed 8,600 women and 5,049 men in a retirement community for 3 to 7 years, during which 418 hip fractures occurred [125]. The risk of a hip fracture was not different across tertiles of dietary calcium intake, ranging from less than 280 mg/day to more than 501 mg/day. Cummings et al followed 9,516 white women for 4 years, during which 192 first hip fractures occurred [38]. Dietary calcium intake (mean 713 mg/day) was not associated with the risk of a hip fracture, and hip fracture risk was not increased in the 11% of women with a dietary calcium intake of 400 mg/day or less. In a second report by these authors in the same group of women (this time followed for 7 years during which 1,950 non-vertebral and 389 vertebral fractures occurred), dietary calcium intake was not associated with fracture risk [39]. Feskanich et al followed 72,337 postmenopausal women for 18 years, during which 603 incident hip fractures occurred [40]. Dietary calcium intake (mean 730 mg/day), total calcium intake and milk consumption were not associated with hip fracture risk. Meyer et al studied 19,752 women and 20,035 men for 11 years during which 213 hip fractures were identified [126]. There was no association between dietary calcium intake (mean 589 mg/day in women and 834 mg/day in men) and the risk of a hip fracture. Benetou followed 10,538 men and 18,584 women from five European countries for 8 years, during which 275 incident hip fractures occurred [127]. Mean dietary calcium intakes by country and sex ranged from 736 mg/day in females in Sweden to 1082 mg/day in males in the Netherlands. There was no association between dietary calcium or dairy intake and the risk of a hip fracture. Michaelsson et al studied 60,689 postmenopausal women from the Swedish Mammography Cohort [128]. During an average of 11 years follow-up, 3,986 women sustained a fracture at any site and 1,535 women a hip fracture. There was no association between dietary calcium intake and the risk of a hip or any osteoporotic fracture. Women with dietary calcium intakes of less than

400 mg/day and more than 1200 mg/day had a similar risk of hip fracture as those with intermediate calcium intakes. Owusu et al studied 43,063 men for 8 years during which 201 forearm and 56 hip fractures occurred [129]. There was no association between total calcium intake (ranging from less than 512 mg/day to more than 1227 mg/day), or milk consumption, and the risk of a fracture. Finally, no relationship between current and lifetime calcium intake and fracture risk over 3 years was reported by Nieves et al among postmenopausal women in the National Osteoporosis Risk Assessment study [114].

In contrast, some studies have reported an inverse association between calcium intake and fracture risk. Holbrook et al followed 1,957 men and women for 14 years during which 33 hip fractures occurred [130]. Dietary calcium intake, when considered as nutrient density (mean 391 mg per 1000 kcal), was inversely associated with hip fracture risk, although the number of fractures was small. Looker et al examined hip fracture risk in a cohort of 4,342 white men and postmenopausal women [131]. During 16 years, 44 men and 122 women experienced a hip fracture. In men, there was no relationship between calcium intake and hip fracture risk (although the risk appeared to be nonsignificantly lower at intakes above 405 mg/day). In the total group of women, there was no relationship between dietary calcium intake and hip fracture risk. When only women who were at least 6 years post-menopause and did not use hormone therapy were considered, the risk of a hip fracture was nonsignificantly lower by almost 50% in the fourth quartile (more than 756 mg/day) compared with the first (less than 292 mg/day). Warensjo et al carried out a recent analysis of the Swedish Mammography Cohort, with an average follow-up period of 19 years, during which 14,738 women sustained a first fracture at any site and 3,871 a first hip fracture [77]. A nonlinear relationship between total calcium intake and risk of fracture was observed. After adjusting for multiple covariates including age, BMI and energy intake, women in the first quintile of dietary calcium intake (less than 751 mg/day) had a significant 18% increased risk of a first total fracture, and a significant 29% increased risk of a first hip fracture, compared with those in third quintile (882 – 996 mg/day). The risk of a total fracture was not further decreased at higher intakes, and the risk of a hip fracture was significantly increased by 19% in the fifth quintile (more than 1137 mg/day) compared with the third.

Bischoff-Ferrari and colleagues have meta-analysed prospective cohort studies of calcium or milk intake and hip fracture risk. They first examined the relationship between total and dietary calcium intake and hip fracture risk in 7 studies comprising 170,991 women who sustained 2,954 hip fractures, and 5 studies comprising 68,606 men who sustained 214 hip

fractures [72]. Total calcium intake was not associated with hip fracture risk among women or men. Their second analysis examined the relationship between milk intake and hip fracture risk in 6 studies comprising 195,000 women who sustained 3,574 hip fractures and 3 studies comprising 75,000 men who sustained 195 hip fractures [132]. Similarly, no relationship between milk intake and hip fracture risk in men or women was reported.

In summary, most studies have found no association between dietary calcium intake and bone loss [83-91], although some have reported an inverse relationship [92-96]. If higher dietary calcium intakes do reduce bone loss, then differences in the rate of loss should compound over time and translate into clinically important differences in BMD and fracture risk in older adults. While most cross-sectional studies do report some association between dietary calcium intake and BMD, the differences are small (for example, a 0.4 – 0.6% higher BMD per 300 mg/day increase in dietary calcium [97-99]). Importantly, most studies have found no association between dietary calcium intake and fracture risk [38-40, 72, 114, 124-126, 128, 129, 132], consistent with a null relationship between calcium intake and bone loss. The relationship between dietary calcium intake and BMD is complicated by the often inconsistent modification by sex [79, 101, 102, 107], weight [102, 103], physical activity [105, 106], smoking [95] and vitamin D status [107]. Furthermore, any relationship appears to be stronger for dairy rather than non-dairy sources of calcium [82, 101]. This could suggest the calcium in dairy is more bioavailable, or that other factors associated with high dairy intakes are responsible for the observed effects. Some trials using interventions with large doses of calcium from food have demonstrated reductions in bone loss [115, 116, 118, 119, 123]. However, due to the size of the calcium dose administered (an additional 600 – 1200 mg/day of calcium), and the use in most trials of foods fortified with additional calcium, these effects may be more similar to that of calcium supplements rather than long-term high dietary calcium intakes.

Calcium supplements and bone health

The effect of calcium supplementation on BMD and fracture risk has been assessed in a number of RCTs. In 2007, Tang et al carried out the most comprehensive meta-analysis of these trials to-date, including a total of 29 RCTs [37]. Data on fracture outcomes were available from 17 trials and 52,625 participants, and data on BMD outcomes from 23 trials

and 41,419 participants. Most participants were women (92%) and their mean age was 68 years. In trials reporting fracture as an outcome, calcium with or without vitamin D treatment was associated with a significant 12% reduction in total fracture risk. This was not different when trials which gave calcium only or calcium with vitamin D were compared. In trials reporting BMD as an outcome, treatment was associated with a reduced rate of bone loss of 0.5% at the hip and 1.2% at the spine. The effect of treatment on fracture was better in trials in which the compliance was higher, which gave doses of at least 1200 mg/day compared with less than 1200 mg/day, and in which the mean dietary calcium intake was less than 700 mg/day compared with more than 700 mg/day (this finding was the same when cut-off points of 1000-1200 mg/day were used). Of the 29 trials included, only 5 examined doses of calcium of less than 800 mg/day.

In most of the larger trials included in this meta-analysis beneficial, but small and nonsignificant, effects of treatment on fracture risk were reported. An exception was a trial by Chapuy et al [12], in which calcium with vitamin D treatment for 18 months significantly reduced hip fracture risk by 43% and nonvertebral fracture risk by 32%. The between-groups difference in total hip BMD at 18 months was 7.3%, an effect size which has not been replicated in other trials of calcium or vitamin D to-date. The large beneficial effects in this study might be related to the population in which it was performed: elderly women aged 69 to 106 years, living in institutions. Dietary calcium intakes and 25-hydroxyvitamin D in these participants were low at baseline (mean 500 mg/day and 40 nmol/l). Subsequently, it has been shown that the vitamin D assay used by Chapuy over-estimated 25-hydroxyvitamin D, and mean levels in the placebo group during the study were approximately 14 nmol/l [133]. El-Desouki have shown that in vitamin D deficient (25-hydroxyvitamin D less than 25 nmol/l) women with osteomalacia, treatment with vitamin D for 12 months increased spine BMD by 51% and femoral neck BMD by 16% [134]. It thus seems certain that some of the women in the trial by Chapuy will have had osteomalacia, and that their treatment would have contributed to the large differences in BMD and fracture risk. For this reason, the findings of this trial are not generalisable to the community-dwelling population. The findings of this trial, and of a second study by these investigators in a similar group of institutionalised women [135], were influential in the findings of the meta-analysis by Tang et al to some degree, particularly the subgroup analyses.

Since the publication of this meta-analysis three trials examining low-dose calcium supplements have been published. Reid et al randomised 323 men to 1200 mg/day of

calcium, 600 mg/day of calcium or a placebo [62]. After 2 years, BMD increased at the lumbar spine, total hip and total body by between 1.0 – 1.5% in the 1200 mg/day group, but did not change in the 600 mg/day group or the placebo group. Nakamura et al randomised 450 Japanese women to 500 mg/day of calcium, 250 mg/day of calcium or a placebo [91]. After 2 years, women in the 500 mg/day group had a 1.2% smaller decrease in BMD at the spine compared with the placebo group. There was no significant difference in bone loss in the 250 mg/day group compared with the placebo group. Chailurkit et al randomised 336 postmenopausal Thai women to 500 mg/day of calcium or a placebo [136]. After 2 years, lumbar spine BMD increased by 2.8% in the calcium group and did not change in the placebo group. Femoral neck BMD did not change in the calcium group and declined by -0.9% in the placebo group.

The meta-analysis by Tang et al examined total fracture as an endpoint [37]; however the effect of calcium supplements might be different by fracture type. In their trial of postmenopausal women, Reid et al reported nonsignificant but downward trends in vertebral, forearm and total fractures with calcium treatment, but a significant *increase* in hip fractures [137]. To determine whether this effect was seen in other trials of calcium supplements, Reid and colleagues repeated the Tang meta-analysis, considering only hip fractures as an outcome [138]. As I have already described, the effects of calcium and vitamin D on fracture risk may be different in the institutionalised population compared with the general community-dwelling population. Therefore, the effects of calcium with vitamin D on hip fractures in trials in institutionalised and community-dwelling populations were analysed separately. In the community-dwelling population, calcium monotherapy nonsignificantly increased hip fracture risk by 61% ($p = 0.099$) and calcium with vitamin D treatment had no effect on hip fracture risk. In contrast, in the institutionalised population, calcium with vitamin D treatment significantly reduced the risk of hip fracture by 25%.

In summary, calcium supplementation with or without vitamin D reduces the rate of bone loss by 0.5% at the hip and 1.2% at the spine, and reduces total fracture risk by around 12%. For hip fractures, calcium supplementation with or without vitamin D has a null or possibly harmful effect on risk in the community dwelling-population. However, in the elderly, institutionalised and vitamin D deficient population, calcium with vitamin D appears to reduce hip fracture risk by 25%.

Mechanism by which calcium intake influences bone health

A calcium intake below that required to replace obligatory calcium losses will result in negative calcium balance and bone loss. In early calcium balance studies, the intake required for neutral calcium balance in postmenopausal women was suggested to be 1500 mg/day [10]. As dietary calcium intakes are much lower for most people, calcium deficiency is assumed to be widespread, and to play some role in the pathogenesis of osteoporosis. The positive effects of calcium supplements on bone loss and fracture risk in RCTs are generally attributed to the correction of this dietary calcium deficiency. However, inconsistent with a dietary calcium deficiency, in most observational studies no association between dietary calcium intake and bone loss [83-91] or fracture risk [38-40, 72, 114, 124-126, 128, 129, 132] is found. Furthermore, in a cross-sectional study in adults, PTH was not associated with dietary calcium intake (except among those who were markedly vitamin D deficient) [139]. Thus, there is no clear evidence that a calcium deficiency occurs within the normal range of calcium in the diet. Consistent with this, Hunt and Johnson reported a tight control of calcium balance at intakes between 400 – 1700 mg/day in a recent analysis of calcium balance data [58].

There are several reasons why calcium supplementation and high intakes of dietary calcium may have a different relationship with bone health. Firstly, calcium supplements are likely result in a greater acute elevation in serum calcium than calcium from the diet, and thus result in greater suppression of PTH and bone resorption. Calcium supplements are taken in one or two bolus doses of 500 – 1000 mg each day, which acutely increase serum calcium and suppress PTH [68-71]. In contrast, dietary calcium will be consumed in small amounts, spread over a day. Even large doses of calcium from the diet appear to have a smaller calcaemic effect than calcium from supplements [140, 141], although this is less well studied. This could be related to the protein and fat in dietary calcium sources, which may slow digestion and the rate at which calcium is released into the blood. Alternatively, the presence of phytates and oxalates in dietary sources of calcium such as spinach and wholegrain cereals might interfere with its absorption [142, 143]. Secondly, calcium supplementation results in a *change* in calcium intake of ~1000 mg/day whereas dietary calcium intake may have remained more stable for years. In order to maintain neutral calcium balance, this change in intake must be adapted to through changes in calcium absorption, calcium excretion and at least initially, osteoclastic bone resorption. Correspondingly, the effects of calcium

supplements on BMD are greatest in the 1 – 3 years after calcium supplementation is initiated and minimal or nil thereafter [14, 137]. Finally, calcium supplementation results in the addition of an isolated micronutrient to the diet. In contrast, higher dietary calcium intakes, which usually reflect higher dairy intakes, will be associated with higher intakes of other nutrients [144]. These co-ingested nutrients might alter the effects of the additional calcium on calcium metabolism. For example, protein increases the absorption of calcium but also increases its excretion [145] and phosphate complexes with ionised calcium and stimulates PTH secretion [146]. Dairy products also contain many bioactive compounds, such as lactoferrin and fatty acids, which may alter the effects of dairy products versus supplemental calcium on calcium and bone metabolism [147, 148].

Summary of calcium and bone health

In summary, high intakes of calcium, whether through diet or supplements, have been widely encouraged for bone health. In clinical trials, calcium supplements modestly reduce bone loss and total fracture risk. In contrast, most observational studies have found no association between dietary calcium intake and bone loss or fracture risk. There are several reasons why calcium supplements and dietary calcium may have different effects on bone, perhaps involving different effect on serum calcium or related to the change in calcium intake that occurs with the initiation of supplementation.

As I will describe in the following section, the cardiovascular safety of calcium supplements has recently been called into question. This has resulted in dietary calcium being increasingly recommended as the preferred source of calcium for bone health [31, 32, 149]. However, dietary calcium intakes often fall below the recommended levels [22, 63, 64], and increasing calcium intake without the use of supplements is likely to be difficult. As efforts to improve bone health should be evidence-based, understanding the relationship between dietary calcium and bone health is important.

2.3 CALCIUM AND CARDIOVASCULAR HEALTH

High dietary calcium intakes and calcium supplementation have in the past been suggested to positively influence cardiovascular health. In observational studies, dietary calcium intake has been associated with lower blood pressure [150], improved lipids [151] and lower body weight [152]. In clinical trials, calcium supplementation has been associated with small reductions in blood pressure [153-156], as well as reductions in weight [152] and improvements in lipids [157], although this has not been consistent [153, 158, 159]. In contrast, in patients with renal impairment, the use of calcium as a phosphate binder clearly increases the risk of vascular calcification and mortality [160-162]. However, this risk was not thought to apply to the general population. Recently, the cardiovascular safety of calcium supplements in the general population has been called into question, with a meta-analysis of RCTs of calcium supplements demonstrating an increased risk of myocardial infarction and stroke [29]. Unsurprisingly, these negative findings have generated ongoing debate in the scientific literature. The following section will review the evidence relating to supplemental and dietary calcium intake and cardiovascular risk, and discuss how calcium intake might influence this outcome.

Intervention studies

The first evidence of an adverse cardiovascular effect of calcium supplements in the general population came from a study by Reid, Bolland and colleagues [137, 163]. This was a trial of 1,471 postmenopausal women randomised to 1000 mg/day of calcium or a placebo for 5 years, with a primary endpoint of fracture incidence. Because beneficial effects on blood pressure and lipids had been observed in previous trials, cardiovascular events were a prespecified secondary endpoint, with the hypothesis that calcium might reduce such events. Contrary to this hypothesis, analysis of adjudicated events in this trial revealed allocation to calcium was associated with a non-significant 49% increase in the risk of myocardial infarction, 37% increase in the risk of stroke and 21% increase the composite endpoint of myocardial infarction, stroke and sudden death [163]. This trial therefore revealed concerning upward trends in cardiovascular events with calcium treatment, which due to the size of the trial and borderline significance, were not definitive.

To determine whether these trends were found in other trials, Bolland et al carried out a meta-analysis of RCTs of calcium monotherapy [164]. As it was possible vitamin D might influence cardiovascular risk, trials in which calcium was coadministered with vitamin D were excluded. Cardiovascular events were not a primary endpoint in any of the included trials, nor a prespecified endpoint of most trials, and events were therefore identified from adverse event databases. Patient-level data on cardiovascular events were available from 5 trials and 8,151 participants, and trial-level data from 11 trials and 11,921 participants. In the patient-level analysis, 143 people allocated to calcium had a myocardial infarction over a follow-up period of 3.6 years, compared with 111 people allocated to placebo, which translated into a significant 31% increase in risk. Similarly, 167 people allocated to calcium had a stroke, compared with 143 people allocated to placebo, which translated into in a non-significant 20% increase in risk. Importantly, the size and direction of the risk were consistent across all of the larger trials included in this meta-analysis.

These findings contrasted with that of the Women's Health Initiative (WHI) trial, in which cardiovascular events were a prespecified endpoint. In the original analyses of this trial of more than 36,000 women, calcium with vitamin D treatment for 7 years was not associated with the risk of myocardial infarction or coronary heart disease death, or with the risk of stroke [165]. The different findings of the meta-analysis of calcium monotherapy trials and the WHI might be explained by the coadministration of vitamin D in the WHI. The participants in the WHI were also younger and more obese than those in the meta-analysis. Alternatively, an adverse cardiovascular effect may have been present in the WHI, but undetected, due to the unusual study design. Participants in this study were permitted to use personal, non-protocol calcium and vitamin D supplements throughout the trial, with 54% of participants reporting personal calcium supplement use at randomisation. The findings of this trial would have therefore largely represented the effect of changing existing calcium supplementation, rather than initiating calcium supplementation.

To determine whether the high rate of personal calcium supplement use in the WHI obscured an effect of trial allocation to calcium with vitamin D, Bolland et al performed a test for an interaction between personal calcium supplement use at randomisation and the effect of treatment allocation on cardiovascular risk, using the publically available WHI dataset and a protocol approved by National Heart Lung and Blood Institute [29]. Significant interactions were identified for clinical myocardial infarction, stroke and their composite endpoint. Among participants *not* using personal calcium supplements, allocation to calcium with

vitamin D significantly increased the risk of clinical myocardial infarction by 22%, and was associated with upward but non-significant trends in stroke and the composite endpoint of clinical myocardial infarction and stroke. In those using personal calcium supplements, allocation to calcium with vitamin D did not increase cardiovascular risk.

This re-analysis of the WHI indicated that the increased cardiovascular risk associated with calcium supplementation is similar whether or not vitamin D is co-administered. Bolland et al therefore repeated their meta-analysis of RCTs of calcium supplements, including data from participants in the WHI not using personal, non-protocol supplements, as well as from two further trials where calcium was coadministered with vitamin D [29]. Patient-level data were now available from five trials on 24,869 participants, with a mean follow-up period of 5.9 years, and trial-level data from 8 trials on 28,072 participants, with a mean follow-up period of 5.7 years. In the patient-level analysis, calcium with or without vitamin D treatment was associated with a significant 26% increase in the risk of myocardial infarction and 19% increase in the risk of stroke. Again, the increased risk was consistent across all of the larger trials.

Since the publication of these meta-analyses, one of the included trials has published new data on cardiovascular outcomes [166]. Self-reported myocardial infarction and stroke events from this trial had been included in the two meta-analyses by Bolland et al [29, 164]. The investigators in this trial subsequently published non-adjudicated hospital discharge data for a new composite endpoint of 'atherosclerotic vascular mortality or first hospitalisation', which included diverse diagnoses such as heart failure and arrhythmias. They reported that allocation to calcium was not associated with the risk of this composite endpoint. Since the findings of the meta-analyses suggest the adverse effects of calcium supplementation relate to myocardial infarction and stroke, including other unrelated diagnoses in this endpoint will obscure any effect of calcium. The reported adjusted hazard ratio (HR) for this composite endpoint was 0.94 (0.69, 1.28), the upper confidence limit of which includes the increase in myocardial infarction and stroke risk suggested in the meta-analyses by Bolland.

One further trial of calcium supplements, this time in combination with sunlight exposure, has reported mortality outcomes. Sambrook et al randomised 602 elderly residents of rest homes (mean age 86 years) to 30 - 40 minutes/day of sunlight exposure only, sunlight exposure with 1000 mg/day of calcium or to a control group who received no intervention [167]. Cardiovascular events were not a prespecified endpoint of this trial; however due to

the age of participants, one-third of participants had died after 2.4 years of follow-up. Compared with the group who received sunlight alone, the group who received sunlight and calcium had a 48% increased risk of all-cause mortality, a 76% increased risk of cardiovascular disease mortality, a 439% increased risk of myocardial infarction mortality and a non-significant 32% increased risk of stroke mortality, based on death certificate data [149]. In contrast, the group who received sunlight exposure alone had a non-significantly reduced risk of mortality compared with the control group.

The meta-analyses by Bolland et al [29, 163] have included all RCTs of calcium supplements, with or without vitamin D, with cardiovascular event data available. The findings of these meta-analyses have received criticism, primarily because they represent secondary analyses of RCTs and as such, cardiovascular events were not collected in a standardised manner (for a discussion see [149]). Nonetheless, it is unlikely a trial large enough to refute the findings of these meta-analyses will be carried out in the foreseeable future. Such a trial, with a primary endpoint of harm, would be unethical and difficult to recruit, and would need to be very large. The findings of these meta-analyses will therefore continue to represent the best evidence available for the effects of calcium supplements on cardiovascular risk.

Longitudinal studies

The relationship between dietary and supplemental calcium intake and cardiovascular risk has also been examined in observational studies. As I have already described for bone health, the findings of such studies should be interpreted cautiously, as even with careful correction for baseline differences, the risk of residual confounding remains. High intakes of dietary calcium are associated with several factors which might influence cardiovascular health including age, smoking status, alcohol consumption, physical activity and socioeconomic status [36, 77-79]. High calcium intakes are usually reflective of high dairy intakes, and high dairy intakes are associated with higher intakes of a range of nutrients, including protein, saturated fat, vitamin D, folate and vitamin E [80-82]. Studies which have compared calcium supplement users and nonusers are also at risk of confounding, as it has been repeatedly shown that users of medicines, such as hormone replacement therapy, are different to non-users [168]. In the re-analysis of the WHI by Bolland et al [29], women who chose to take personal calcium supplements were different from women who did not use personal

supplements in age, BMI, blood pressure, use of hormone replacement therapy, history of myocardial infarction or stroke and smoking status. Evidence from observational studies is therefore of lesser value for the effect of calcium supplements on cardiovascular outcomes, as clinical trial data from almost 30,000 participants is available [29]. However, evidence from prospective cohort studies will represent the best available for the effect of dietary calcium on cardiovascular outcomes, as similar to fracture risk, evidence from clinical trials is not available.

Since the publication of the first trial in which a possible adverse cardiovascular effect of calcium supplements was reported [163], several investigators have utilised databases from prospective cohort studies to examine relationships between calcium supplement use and /or dietary calcium intake and cardiovascular risk. Pentti et al followed 10,555 women for 7 years during which 513 women were diagnosed with coronary heart disease [169]. The risk of coronary heart disease was increased by 24% in calcium supplement users versus nonusers. Li et al followed 23,980 men and women for 11 years during which 354 myocardial infarctions, 260 strokes and 267 cardiovascular disease deaths occurred [36]. Compared with those in the first quartile of dietary and dairy calcium intake, those in the third quartile had a 30% lower risk of myocardial infarction. In contrast, compared with nonusers of any supplements, users of calcium supplements had an 86% increased risk of myocardial infarction, which increased to 139% for users of calcium-only supplements. Dietary and dairy calcium intakes, and calcium supplement use, were not associated with the risk of stroke or cardiovascular death. Xiao et al followed 388,229 men and women for 12 years, during which 7,904 cardiovascular disease deaths occurred among men and 3,874 among women [170]. Dietary calcium intake was not associated with cardiovascular disease death in men or women. In men, those consuming 1000 mg/day or more of supplemental calcium had a 20% increase in the risk of cardiovascular disease death compared with nonusers of calcium supplements. A smaller and non-significant increase in risk was observed in women.

In two further recent studies no effect of supplemental calcium intake on cardiovascular risk was found, although in one there was an association with dietary calcium. Michaelsson et al followed 61,433 women for 19 years during which 11,944 deaths from all-causes, 3,862 deaths from cardiovascular disease, 1,932 deaths from ischaemic heart disease and 1,100 deaths from stroke, occurred [171]. A non-linear relationship between dietary calcium intake and mortality was observed. Compared to those with a dietary calcium intake of 600 to 1000

mg/day, those with intakes of 1400 mg/day or more had a 40% increased risk of all-cause mortality, a 49% increased risk of cardiovascular disease mortality, and a 114% increased risk of ischaemic heart disease mortality. Dietary calcium intake was not associated with the risk of stroke mortality. Calcium supplement use was not associated with the risk of mortality, except among women with a dietary calcium intake of 1400 mg/day or more, in whom calcium supplement use was associated with 157% increase in the risk of all-cause mortality. Van Hemelrijck followed 20,024 men and women from 1988-1994 through to 2006 during which 10% died from cardiovascular disease [172]. Dietary or supplemental calcium intake was not associated with the risk of cardiovascular disease death.

Prior to the first report of an adverse cardiovascular risk associated with calcium supplements [163], the relationship between calcium intake and cardiovascular risk had been examined in several large observational studies, all of which had reported a null or beneficial relationship. Bostick et al followed 34,486 postmenopausal women for 8 years during which 348 deaths from ischaemic heart disease occurred [173]. The risk of dying from ischaemic heart disease was reduced by a third among those in the highest quartile of total calcium intake compared with the lowest. The same reduction in risk applied when only dietary or supplemental calcium intakes were considered. Al-Delaimy followed 39,800 men for 12 years during which 1,548 cases of ischaemic heart disease were documented. Total calcium intake, dietary calcium intake and supplemental calcium intake were not associated with the risk of ischaemic heart disease [174]. Umesawa et al followed 110,792 Japanese men and women for 10 years, during which there were 1,329 deaths from cardiovascular disease and 264 deaths from coronary heart disease [175]. Dietary calcium intake was not associated with mortality from coronary heart disease or total cardiovascular disease. Dairy calcium intake was inversely associated with mortality from total cardiovascular disease. In a second cohort of 41,526 Japanese men and women [176], Umesawa reported that dietary calcium intake was not associated with the risk of coronary heart disease over 13 years of follow-up.

A number of studies have reported calcium intake in relation to stroke risk. Larsson et al recently carried out a dose-response meta-analysis of these studies, which included 9,095 stroke events from 11 studies [177]. Evidence of a nonlinear association between dietary calcium intake and stroke was reported, with an inverse association only at lower calcium intakes. When analyses were stratified by the average calcium intake in the study population, the risk of stroke was decreased by 18% for a 300 mg/day increase in dietary calcium intake in populations with a low calcium intake (less than 700 mg/day), but in populations with a

high calcium intake (700 mg/day or more) the corresponding risk was weakly but significantly increased by 3%. Supplemental calcium intake was not associated with the risk of stroke in the three studies which examined calcium from supplements.

In summary, in meta-analyses of RCTs, calcium supplementation is associated with a significant increase in the risk of myocardial infarction and stroke [29, 164]. Findings from observational studies have been more inconsistent, with some studies reporting a direct association between calcium supplement use and cardiovascular risk [36, 169, 170], while others have reported no association [171, 172, 174, 177] or even an inverse association [173]. For dietary calcium intake, most observational studies have found no association with cardiovascular risk [170, 172, 174-176] or an inverse association [36, 173, 177], with the exception of one study where an increased risk of cardiovascular mortality was observed at dietary calcium intakes of greater than 1400 mg/day [171]. Therefore, dietary calcium does not appear to be associated with the same adverse cardiovascular risk as calcium supplements, although the evidence for dietary calcium is of lesser quality.

Mechanism by which calcium intake influences cardiovascular health

The mechanism by which calcium supplements increase cardiovascular risk is presently unclear. As most evidence suggests dietary calcium does not increase cardiovascular risk, differences in the physiological effects of calcium supplementation and high intakes of dietary calcium might help to identify this mechanism. The following section will discuss the differences between supplemental and dietary calcium, and as I have already described for their effects on bone health, how these differences might relate to cardiovascular health.

Firstly, the adverse cardiovascular effects of calcium supplements might be mediated through increased serum calcium. Calcium supplements are likely to have a greater impact on serum calcium than dietary calcium, as they are consumed in large boluses, once or twice a day, which acutely elevate serum calcium [68-71, 178]. In contrast, dietary calcium will be consumed in smaller amounts spread over a day; while even large doses of calcium from food may have a smaller calcaemic effect than equivalent doses of supplemental calcium [140, 141]. Increased serum calcium concentrations, within the normal range, have been associated with cardiovascular risk in cohorts. In a study of 2,183 men, Lind et al reported that a higher serum total calcium (2.37 versus 2.35 mmol/l) was associated with an increased risk of

myocardial infarction [179]. In a study of 12,865 men and 14,293 women, Jorde et al reported that a 0.10 mmol/l increase in serum calcium was associated with a 20% increase in the risk of myocardial infarction in men, and with a similar but nonsignificant increase in women [180]. In a study of 2,176 men, Larsson et al reported that a 0.09 mmol/l increase in serum calcium increased the risk of total mortality by 8% and non-significantly increased the risk of cardiovascular mortality by 8% [181]. In a study of 7,259 postmenopausal women, Slinin et al reported that a 0.09 mmol/l increase in total calcium was associated with a 17% increase in the risk of a cardiovascular event [182]. In contrast, in a study of 3,368 men and women, Dhingra et al reported no association between serum calcium and the risk of cardiovascular disease [183]. In a study of 20,024 men and women, van Hemelrijck reported that the risk of cardiovascular disease was increased for those in the lowest 5% of serum ionised calcium (<1.16 mmol/l) compared with the mid 90% [172]. Among women, the risk of ischaemic heart disease death was increased in the upper 5% of ionised calcium (>1.31 nmol/l), while among men, the risk of ischaemic heart disease death was increased in the lowest 5% of ionised calcium.

Secondly, calcium supplementation results in a change in calcium intake of 1000 mg/day, while dietary calcium intakes may have remained more stable for years. This abrupt change in intake appears to require a period of adaption before calcium balance is restored to neutral. For example, Spiegal et al compared the effects of short-term medium calcium (800 mg/day) versus high calcium (2000 mg/day) diets on calcium balance in normal participants and patients with chronic kidney disease (CKD) [51]. After 9 days on the medium calcium diet, all participants were in a slight negative to neutral calcium balance. After 9 days on the high calcium diet, the CKD patients were in a positive calcium balance of +750 mg/day and the normal participants in a positive calcium balance of +460 mg/day. Presumably this positive calcium balance is not maintained long-term, as it would predict a doubling in total body calcium over several years. Nonetheless, it is possible that a period of positive calcium balance might have some adverse effect on cardiovascular risk.

Finally, calcium supplementation results in the addition of a large amount of an isolated micronutrient to the diet. In contrast, higher dietary calcium intakes, which usually reflect higher dairy intakes, will be associated with higher intakes of other nutrients [144]. High dairy intakes are associated with higher intakes of calcium as well as protein, magnesium, potassium, zinc, sodium, folate, thiamin, riboflavin, and vitamins A, D, B-12, B-6 and E [80, 81]. Higher intakes of nutrients other than calcium might explain the null or protective effects

of a high dietary calcium intake on cardiovascular risk. There is also the possibility that some nutrients might directly modify the physiological effects of calcium. Magnesium, for example, acts in several respects as a physiological antagonist of calcium [184], and high intakes of magnesium and high serum magnesium are associated with a reduced risk of cardiovascular events [185]. Furthermore, dairy products contain a number of bioactive compounds, such as casein phosphopeptides, which may have beneficial effects on blood pressure and blood coagulation, and could therefore influence the effects of a high dairy intake on cardiovascular health [186, 187].

Due to the diverse range of biological processes calcium participates in, there exist a number of ways in which the large and sustained increase in calcium intake that occurs with calcium supplementation, or the associated acute increases in serum calcium, might influence cardiovascular risk. The following sections will review the effects of supplemental, dietary and/or serum calcium on the following indices of cardiovascular disease: blood pressure, blood coagulation and regulators of vascular calcification.

Blood pressure

Perhaps the most widely studied cardiovascular effect of calcium supplements is that on blood pressure. In observational studies, higher intakes of dietary calcium have been inversely associated with blood pressure [188-192]. In a meta-analysis of observational studies, an increase in dietary calcium of 100 mg/day was associated with a reduction in systolic and diastolic blood pressure of 0.4 mmHg [150]. Similarly, in meta-analyses of clinical trials, calcium supplementation is associated with reductions in systolic and diastolic blood pressure of 0.8 – 2.5 mmHg [154-156]. In contrast, in the WHI, calcium with vitamin D treatment had no effect on blood pressure or the risk of hypertension over 7 years [193]. This may have been related to the design of the trial (as I have described for cardiovascular events), or to the duration of treatment. In another of the largest and longest trials to examine the effects of calcium supplements on blood pressure, Reid et al reported that the greatest antihypertensive effects of calcium were observed after 6 months, and were minimal thereafter [159].

In contrast, a separate body of evidence suggests increased serum calcium has a hypertensive effect. In healthy individuals, a calcium infusion which increased ionised calcium by 0.32 mmol/l increased systolic blood pressure from 114 to 121 mmHg [194]. Similarly, a calcium infusion which increased ionised calcium by 0.20 mmol/l increased systolic blood pressure from 123 to 134 mmHg [195]. Earlier studies using intravenous infusions of calcium have consistently reported elevations in blood pressure associated with hypercalcaemia [196-199]. Further evidence comes from studies which have examined the effect of the dialysate calcium concentration on blood pressure in patients undergoing hemodialysis. Following the use of a low calcium (1.25 mmol/l) versus high calcium (1.50 mmol/l) dialysate, post-dialysis ionised calcium concentrations were 1.11 and 1.19 mmol/l, systolic blood pressures 134 mmHg and 145 mmHg and diastolic blood pressures 68 mmHg and 74 mmHg, respectively [200]. Previous studies comparing high and low calcium dialysates have consistently reported less of a reduction in intradialytic blood pressure with the use of higher calcium dialysates [201-203]. These effects have been related to changes in myocardial contractility, stroke volume and/or peripheral resistance [200, 202-206].

Calcium supplementation therefore appears to have a modest and possibly short-term antihypertensive effect, while interventions which increase serum calcium have an acute hypertensive effect. As calcium supplements are known to elevate serum calcium to a modest degree for hours following their ingestion, a possible transient hypertensive effect could have been missed in clinical trials, due to the timing of the blood pressure measurement. In two calcium supplement trials by Reid et al [153, 159], blood pressure was measured the morning after an overnight fast. This may also apply to other trials which have measured blood pressure, although this is not usually described in their methods.

To my knowledge, only two studies, both recent, have reported changes in blood pressure acutely following the ingestion of an oral calcium dose. In an uncontrolled study of 25 men and women, total and ionised calcium increased 3 hours after an oral dose of 1000 mg of calcium citrate by 0.10 mmol/l and 0.06 mmol/l, respectively, but blood pressure did not change from baseline [207]. In a small cross-over study of 11 men and women, albumin adjusted total calcium increased 2 hours after 600 mg of calcium as citrate and calcium from food; however blood pressure did not change [208].. Heart rate decreased after calcium citrate and increased after calcium from food, but no effects on other vascular parameters were observed. The lack of an effect of calcium supplementation on blood pressure acutely in these trials might suggest that the changes in serum calcium were too small to replicate the

effects on blood pressure of a calcium infusion. Their findings are, however, limited by the small number of participants and lack of a calcium-free control. The acute effects of calcium supplements on blood pressure therefore require further investigation.

Blood coagulation

Blood coagulation resulting in thrombosis is the major process leading to myocardial infarction and stroke. Calcium is an essential cofactor for a number of steps in the coagulation cascade, and is necessary for the conversion of pro-thrombin to thrombin, and fibrin to fibrinogen [209]. Furthermore, blood coagulation is dependent on platelet function, and platelets express the calcium-sensing receptor [210]. The inhibitory effects of hypocalcaemia on blood coagulation are well established, but little research has examined the effects of high-normal serum calcium or hypercalcaemia on coagulation. Calcium concentrations above the threshold required for clotting to occur have not thus far been shown to influence coagulation [211]. Furthermore, the widely used laboratory measures of coagulation, prothrombin time and activated partial thromboplastin time, are unsuitable for examining the relationship between serum calcium and coagulation, as they are measured in plasma, involve the flooding of citrated samples with calcium, and only provide a measure of one aspect of the coagulation system.

Thromboelastography (TEG) is a method of measuring clotting in whole blood, and is based on the motion of a pin suspended in a rotating cup containing a blood sample [212]. TEG provides a dynamic measure of *in vivo* haemostasis, reported as the following four parameters: R-time, K-time, alpha-angle and maximum-amplitude. Each parameter is understood to represent an aspect of coagulation system; however exact processes which contribute to each parameter have not been fully elucidated. R-time is a measure of the time to clot initiation and represents initial fibrin formation [212-214]. K-time is a measure of the time for the clot to reach a prespecified level of strength or firmness and is related to the rate of polymerisation of the clot. K-time is shortened by an increased fibrinogen level, and to a lesser extent, increased platelet function. Alpha-angle is a measure of the rate of clot strengthening and is closely related to K-time, as it is also a function of the rate of polymerisation of the clot. Alpha-angle is increased by increased fibrinogen levels, and to a lesser extent, platelet function. Finally, maximum-amplitude is a measure of the maximum

strength or stiffness of the clot, and is related primarily to platelet count and function. A hypercoagulable state will be indicated by a shortened R-time or K-time, or an increased alpha-angle or maximum amplitude; and a hypocoagulable state by changes in the opposite direction. While TEG has not undergone the same evaluation processes as conventional tests of clotting [214], the ability of TEG to detect hypercoagulable states and predict postoperative thrombotic complications, including myocardial infarction, has been demonstrated [215-217].

Few studies have used TEG to examine the effect of the calcium concentration on blood coagulation. In an early study in rats, those with induced hypercalcaemia had a shortened R-time compared with normocalcaemic animals [218]. The administration of EDTA returned R-time to normal. An *in vitro* study used TEG to measure clotting in citrated whole blood samples from human volunteers that had been recalcified with varying amounts of calcium [219]. A significant inverse relationship between ionised calcium and R-time was reported, however this appeared to be strongest between an ionised calcium concentration of 0.60 – 0.80 mmol/l, well below the normal range in blood. In contrast, a similar *in vitro* study demonstrated a V shaped relationship between ionised calcium and R-time and K-time, with minimal values occurring at an ionised calcium concentration of 2.1 mmol/l. These findings suggested that blood became more hypercoagulable up to an ionised calcium concentration of 2.1 mmol/l, far exceeding the normal range in blood. The findings of both studies must be interpreted with caution, however, as coagulation in citrated and recalcified blood samples may not accurately reflect coagulation in whole blood [220, 221]. No study has yet examined the effect of the serum calcium concentration on clotting in native whole blood samples.

Vascular calcification

Increased vascular calcification is perhaps the most obvious way in which calcium supplements might influence cardiovascular risk, as the use of calcium as a phosphate binder is well-established to accelerate vascular calcification in patients with CKD [160-162]. Consistent with this, serum calcium is directly associated with calcification in the general population. In healthy older women, Wang et al reported that the risk of abdominal aortic calcification increased by 23% for every 0.10 mmol/l increase in total calcium [222]. In older men and women, Rubin reported that participants in the top quintile of carotid plaque

thickness were more likely to be in the top quintile of serum total calcium (>2.28 mmol/) [223]. However, an effect of calcium supplementation on calcification risk in the general population has not been demonstrated. In an RCT of calcium supplements, Prince et al reported that common carotid artery thickness and carotid atherosclerosis were not different between the calcium and control groups 3 years into the trial (baseline measurements were not performed) [224]. In a prospective study of 690 women and 580 men, Samelson reported that total calcium intake at baseline was not associated with coronary artery calcification score 4 years later [78]. The findings were the same when only dietary or supplemental calcium were considered. Furthermore, in the meta-analysis of calcium supplements and cardiovascular risk [29], the increase in myocardial infarction risk was apparent from around 1 year of treatment, suggesting its pathogenesis might involve a faster process than that of vascular calcification.

Vascular calcification, like bone mineralisation, is a biologically regulated process under the control of promoters and inhibitors. If calcium supplements did influence the risk of vascular calcification, then this might be mediated through effects on regulators of calcification, rather than by simply increasing the amount of substrate available for precipitation. The following section will briefly describe a method of measuring the propensity of serum to calcify, as well as three known or suspected regulators of vascular calcification: fetuin-A, pyrophosphate and fibroblast growth factor -23 (FGF23), and how these may be influenced by calcium.

Serum calcification propensity

A novel test which measures the overall propensity of serum to calcify was recently described by Pasch and colleagues [225]. This test is based on the time taken for primary calciprotein particles in serum to transition into secondary calciprotein particles, when supraphysiologic concentrations of calcium and phosphate are added. These particles are initially spherical, soluble and amorphous, known as primary calciprotein particles, but eventually transition into elongated, insoluble and crystalline secondary calciprotein particles [226]. A greater transition time (T_{50}) reflects a greater ability of serum to withstand calcification. The ability of T_{50} to predict outcomes in patients with CKD was recently demonstrated [227]. In a prospective study of 184 elderly hypertensive patients, reduced T_{50} was associated with an increased risk of all-cause mortality and with progressive aortic stiffness. In a baseline cross-sectional analysis in this study, reduced T_{50} was associated with lower concentrations of

fetuin-A, magnesium and pyrophosphate, and with higher concentrations of ionised calcium and phosphate [228]. Calcium supplement use was also significantly associated with reduced T_{50} in this study, however the number of calcium supplement users was small (n = 23).

Fetuin-A

Fetuin-A, a plasma protein synthesised in the liver, is a potent inhibitor of vascular calcification, and deficiency of fetuin-A is associated with extensive calcification in mice [229]. The relationship between fetuin-A concentrations and cardiovascular risk appears to depend on the stage of calcification. In end-stage renal disease, lower fetuin-A concentrations are associated with increased cardiovascular mortality [230, 231]. In contrast, in non-dialysed patients with diabetic nephropathy, increased fetuin-A is positively associated with the magnitude of coronary artery calcification [232]; and in the general population, increased fetuin-A is associated with the risk of myocardial infarction and stroke [233]. This might indicate that the synthesis of fetuin-A is initially up-regulated as a defense against the onset or early stages of vascular calcification, and that as the burden of vascular calcification increases, fetuin-A synthesis becomes exhausted [232]. Whether calcium intakes or serum calcium influence fetuin-A in the general population has not been studied. However, the administration of a vitamin-D analog (paricalcitol) to hyperparathryoid patients on dialysis increased fetuin-A after 8 weeks of treatment [234] and in children with CKD, increased serum calcium was positively associated with fetuin-A [235].

Pyrophosphate

Pyrophosphate is a small molecule produced by smooth muscle cells which complexes with calcium, acting as a potent inhibitor of calcification [236]. Low plasma pyrophosphate was associated with an increased calcification score at baseline, and greater change in calcification score over 1 year, in patients with CKD [237]. Although the determinants of the pyrophosphate concentration are not fully understood, alkaline phosphatase cleaves pyrophosphate [238], whereas increased serum phosphate appears to increase pyrophosphate [237, 239]. It is not known whether calcium intake or serum calcium affect pyrophosphate. However, as pyrophosphate complexes with calcium, it is possible that increased serum calcium might bind with and thereby reduce the amount of free pyrophosphate.

Fibroblast growth factor -23

FGF23 is a hormone secreted by osteoblasts which regulates phosphate concentrations by increasing the urinary excretion of phosphate, and inhibiting the renal production of 1,25-dihydroxyvitamin D. Despite its phosphate-regulating role, in patients with CKD, higher concentrations of FGF23 are associated with an increased risk of cardiovascular events [240-242] and mortality [243, 244]. Similarly, in a cohort of community dwelling elderly, higher FGF23 concentrations were associated with impaired vasoreactivity and increased arterial stiffness [245], increased left ventricular mass [246] and atherosclerosis [247]. The regulation of FGF23 is incompletely understood, but calcitriol and phosphate are both known to increase its concentration [248, 249]. In animal studies, calcium has also been shown to regulate FGF23, with dietary calcium supplementation increasing FGF23 [250] and diet-induced hypocalcaemia reducing its concentration [251]. Whether a similar relationship exists in humans has not been studied.

Summary of calcium and cardiovascular health

In summary, calcium supplementation is associated with increased cardiovascular risk, while most evidence suggests dietary calcium is not. There are several reasons why calcium supplements and dietary calcium may have different effects on cardiovascular health, perhaps involving different effects on serum calcium or related to the change in calcium intake that occurs with the initiation of supplementation. Although there exist several possible ways in which calcium supplementation or increased serum calcium might influence cardiovascular risk, perhaps involving changes in blood pressure, blood coagulation or vascular calcification, there is little evidence to document their role. Due to size of the meta-analyses of calcium supplements and cardiovascular risk, a trial large enough to refute their findings is unlikely to be carried out in the foreseeable future. Research is therefore needed to identify how calcium supplements influence cardiovascular risk.

2.4 CALCIUM AND CANCER

A third health outcome that has been suggested to be associated with calcium intake is cancer. Numerous observational studies have examined the relationship between dietary calcium intake and cancer risk. Although it is beyond the scope of this thesis to review these studies, in general, an inverse relationship between dietary calcium intake and the risk of colorectal cancer [252-254] and possibly breast cancer [255, 256], has been reported, but a direct relationship between dietary calcium intake and the risk of prostate cancer [257-259]. However, these findings have not been consistent [260-265]. Despite a possible effect of dietary calcium intake on cancer risk, few RCTs of calcium supplements have examined cancer as an outcome, probably because of the size and duration of study required. This section will review trials of calcium supplements which have reported cancer outcomes.

An influential study on cancer supplements and cancer risk was a fracture incidence trial of 1,179 postmenopausal women by Lappe et al [266]. Participants in this trial were randomised to 1400 - 1500 mg/day of calcium alone, calcium with 1100 IU/day of vitamin D, or a placebo. A total of 50 cancers occurred during 4 years of treatment. Compared with the control group, the risk of total cancer was nonsignificantly lower by 47% ($p = 0.06$) in the calcium only group, and significantly lower by 60% in the calcium with vitamin D group ($p = 0.01$). The authors suggested that calcium with vitamin D supplementation may reduce cancer risk, while calcium supplementation alone has no effect. However, as the risk of cancer was not different between the calcium only and calcium with vitamin D groups ($p = 0.46$), determining which is the critical component of the intervention is not straightforward.

Two trials have examined the effects of calcium supplements on colorectal adenoma recurrence, which is used as a surrogate measure of colorectal cancer risk. Baron et al randomised 930 men and women to 1200 mg/day of calcium or to placebo for 4 years [267]. The risk of having at least one adenoma during the main risk period (between 1 to 3 years post-randomisation) was 15% lower in the calcium group compared with the placebo group. Invasive large-bowel cancer was diagnosed in only four participants during the main risk period. Bonithon-Kopp randomised 665 men and women to 2000 mg/day of calcium, a fibre supplement, or a placebo, for 3 years [268]. The odds of adenoma recurrence were nonsignificantly reduced by 34% in the calcium group and significantly increased by 67% in

the fibre group. One participant in the placebo group was diagnosed with an invasive adenocarcinoma during the trial.

To examine the effect of calcium supplementation on prostate cancer risk, Baron et al used data from men in their colorectal adenoma prevention trial [269]. A total of 672 men received 1200 mg/day of calcium or a placebo for 4 years, and were followed for an average of 10 years. Over the total period, the risk of prostate cancer was not different in the calcium and placebo groups. During the first 6 years (4 years of treatment and 2 years of follow-up) the risk of prostate cancer was significantly reduced by 48% in the calcium group compared with the placebo group.

Avenell et al examined cancer risk in the Randomised Evaluation of Calcium or Vitamin D (RECORD) study, a trial of 5292 older men and women [270]. Participants were randomised to 1000 mg/day of calcium, 800 IU/day of vitamin D, calcium with vitamin D, or a placebo for 2 to 5 years, and followed for 3 years post-intervention. A total of 182 new cancers were diagnosed. Cancer incidence was not significantly different among participants allocated to calcium and those not, or among those allocated to vitamin D and those not.

Finally, in the original analyses of the WHI, calcium with vitamin D treatment had no effect on the risk of colorectal cancer [271], breast cancer [272] or non-melanoma and melanoma skin cancers [273]. However, as I reviewed earlier, the frequent use of personal, non-protocol calcium supplements in the WHI obscured an adverse effect of calcium with vitamin D allocation on cardiovascular outcomes [29]. Bolland et al therefore hypothesised that personal calcium supplement use may have obscured an effect of treatment allocation on other health outcomes, such as cancer. To determine whether this had occurred, they performed a test for an interaction between personal calcium or vitamin D supplement use at randomisation, and the effect of treatment allocation on cancer risk [30]. Significant interactions between personal calcium or vitamin D supplement use and trial calcium with vitamin D allocation were identified for total, breast, invasive breast and colorectal cancers. In women not taking personal calcium or vitamin D supplements, trial calcium with vitamin D allocation significantly reduced the risk of total cancer, breast cancer, and invasive breast cancer by 14 to 20% and nonsignificantly reduced the risk of colorectal cancer by 17%. In contrast, in women taking personal calcium or vitamin D supplements, trial calcium with vitamin D allocation had no effect on cancer risk. As calcium and vitamin D were administered together

in the WHI, it is unclear whether the protective effects on cancer risk were related to calcium, vitamin D or the combination of both agents.

Summary of calcium and cancer

In summary, in a re-analysis of the WHI data, calcium with vitamin D treatment reduced the risk of total cancer, breast cancer and possibly colorectal cancer. As calcium and vitamin D were given together in this trial, it is unknown which agent was responsible for the protective effects. In an earlier trial, calcium supplementation with or without vitamin D reduced the risk of total cancer by half, raising the possibility that calcium supplements may be protective against cancer. However, the number of cancer events in that trial (of Lappe) was small. Furthermore, other trials have reported beneficial effects of calcium supplements on colorectal adenomas and possibly prostate cancer. Further research is thus needed to examine the effects of calcium supplementation on cancer risk.

CHAPTER 3: ACUTE AND 3-MONTH EFFECTS OF CALCIUM SUPPLEMENTS ON SERUM CALCIUM AND MARKERS OF BONE TURNOVER: A RANDOMISED CONTROLLED TRIAL

3.1 INTRODUCTION

Recent evidence suggests calcium supplementation increases cardiovascular risk [30]. In contrast, most evidence suggests high intakes of dietary calcium do not increase cardiovascular risk [36, 170, 174, 274]. A possible difference between calcium supplements and dietary calcium is their effects on serum calcium. Calcium supplements are taken in large boluses which acutely increase serum calcium [68-71], while dietary calcium will be consumed in smaller amounts spread over a day, and would expected to have a smaller calcaemic effect. It may be this elevation in serum calcium which underlies the increase in cardiovascular risk associated with calcium supplements. However, the duration for which serum calcium is elevated after the ingestion of a calcium supplement, and whether this elevation is diminished with continuous supplementation, are unknown.

As I reviewed in Chapter 2, the lack of an association between dietary calcium intake and fracture risk in most observational studies suggests most people are able to obtain adequate calcium through diet alone. There are, however, certain individuals, such as the elderly or those who are unable to or choose not to consume dairy products, who may have very low calcium intakes [64, 275, 276]. As calcium supplementation may be recommended in these individuals, there exists a need for a safe form of supplemental calcium. Microcrystalline hydroxyapatite (MCH), derived from bovine bone, contains calcium and phosphate in the form of hydroxyapatite, as well as collagenous and non-collagenous bone proteins. MCH has previously been shown to result in a smaller increase in serum calcium than conventional supplements [71]. MCH could potentially provide a form of supplemental calcium with a better cardiovascular safety profile than conventional supplements.

To evaluate MCH as a form of supplemental calcium, I carried out a RCT of postmenopausal women which compared the acute and long-term effects of MCH with conventional calcium supplements on serum calcium and bone turnover. There are a number of ways in which MCH preparations may be produced with respect to the degree of protein hydrolysis and granule size so, as a secondary aim, I determined whether differences in processing would

translate into differences in effects on serum calcium or bone turnover. Furthermore, to better understand the calcaemic effects of calcium supplements, I examined their acute effects on serum calcium for up to 8 hours, as most previous trials have not persisted beyond 4 to 6 hours, and whether any acute effects were attenuated with long-term use.

3.2 METHODS

Participants

The participants were 100 women, at least 5 years post-menopause. They were recruited from women who had volunteered for other osteoporosis studies by the Auckland Bone and Joint Research Group, but had been ineligible for those studies because they had normal bone density. Women were excluded if they had a history of cardiovascular disease or a 5-year cardiovascular risk of >15%; a major ongoing systemic illness; had taken any medication known to affect calcium or bone metabolism in the past year; or were currently taking greater than 2,000 IU/day of vitamin D. Women who regularly used more than 100 mg/day of calcium supplements required a 6-month withdrawal period prior to entering the study. The medical history questionnaires of ~1000 women were reviewed, of whom 415 met the inclusion and exclusion criteria and were invited to participate. Women who expressed interest in participating were asked to complete an updated medical history questionnaire, which was reviewed to ensure they still met these criteria. The flow of participants through the study is presented in Figure 3.1.

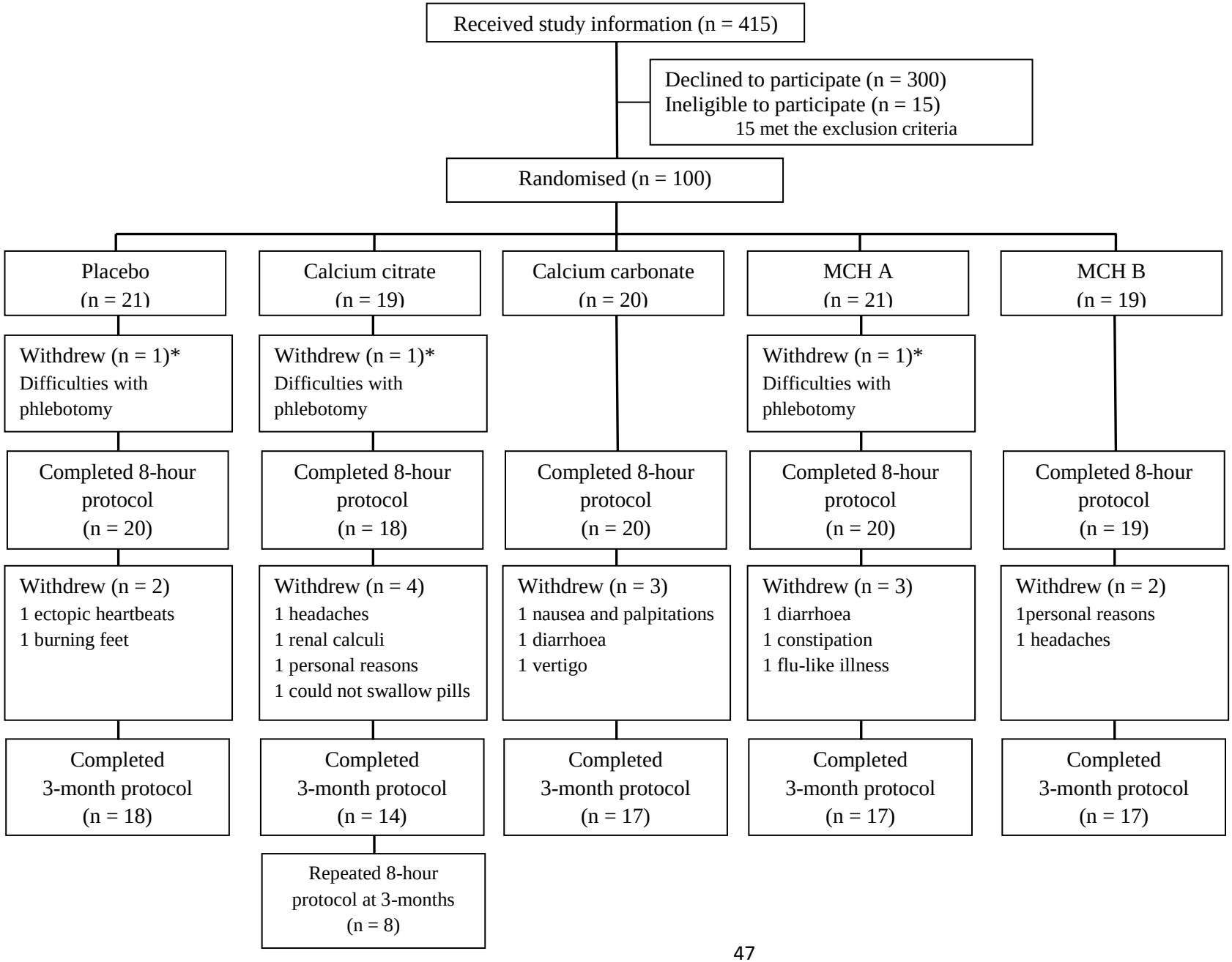

Figure 3.1 Flow of participants through the study. *Withdrew after randomisation but prior to receiving the treatment and did not contribute data to the study.

Study design

Participants were randomised to treatment with calcium (1000 mg/day of elemental calcium) as citrate, carbonate or one of two MCH preparations, or to a placebo containing no calcium, for 3 months. Two MCH preparations (denoted MCH A and MCH B) were supplied by Waitaki Biosciences (Christchurch, New Zealand). The MCH A preparation had a smaller particle size and a greater degree of protein hydrolysis compared with MCH B. Calcium carbonate (Kirsh Pharma, Germany) and calcium citrate (Jost Chemical Co., USA) were purchased from Hawkins Watts (Auckland, New Zealand). All four treatments and placebo were given as powders encapsulated in identical gelatin capsules. Each capsule contained 125mg of calcium, or placebo. Triplicate samples of each of the four treatments and placebo were analysed to verify calcium content. Treatment was randomly assigned using a computer-generated variable-block randomisation schedule prepared by staff not in contact with participants. Participants were blinded to the treatment they received for the duration of the study. Study staff were not blinded throughout the study as only participants allocated to calcium citrate or placebo had certain measurements performed (these results are presented in Chapter 4).

On day 1 of the study, participants attended an 8-hour session at a research clinic after fasting overnight. A baseline blood sample was collected between 0700 and 0900, immediately after which they received the first dose of their allocated treatment with water. Further blood samples were collected 2, 4, 6, and 8 hours after the treatment was ingested. A light breakfast (peaches in juice and toast with margarine and jam, marmalade or honey) was provided immediately after the treatment was ingested; a light lunch (fruit salad in juice and bread with margarine and jam, honey, marmalade or peanut butter) was provided at 4 hours; and an optional snack (plain biscuits and decaffeinated tea) at 6 hours. Meals were provided after the blood sampling was complete for that time-point. The breakfast provided an additional 74 mg of calcium, the lunch 74 to 80 mg of calcium, and the snack 6 mg of calcium, by calculation. Water and non-caffeinated tea without milk were allowed *ad libitum* throughout the day.

After day 1, participants were instructed to take their treatment at home in two divided doses, with their morning and evening meals. Midway through the study, participants were telephoned to encourage compliance. After 3 months, participants returned to the clinic having taken their final dose of treatment the evening before, and a final fasting blood sample

was collected. To determine whether the acute effects of calcium supplements on serum calcium changed after long-term use, participants allocated to calcium citrate were invited to repeat the 8-hour sampling protocol as carried out on day 1. Written informed consent was obtained from all participants. This study received ethical approval from the New Zealand Northern Regional X Ethics Committee (NTX/10/12/125). This study was registered with the Australia New Zealand Clinical Trials registry (ACTRN12611000232932).

Measurements

Ionised calcium was measured on anaerobically handled specimens using an ABL800 FLEX blood gas analyser (Radiometer, Bronshoj, Denmark). The samples for measurement of total calcium and phosphate were batch-analysed at the end of each 8-hour session using a Cobas modular analyser (Roche Diagnostics, Indianapolis, IN). Samples for the measurement of PTH and markers of bone turnover were stored at -70˚C and batch-analysed at the completion of the study. Roche autoanalysers were used for the measurement of PTH, serum procollagen type-I N-terminal propeptide (PINP) and serum C-telopeptide (CTX) (Roche Diagnostics, Indianapolis, IN). Baseline 25-hydroxyvitamin D was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (ABSciex API 4000).

Body weight was measured using electronic scales, and height using a Harpenden stadiometer. Dietary calcium intake was assessed using a validated food frequency questionnaire [277]. Compliance was assessed at the final visit by capsule count and calculated as the number of capsules taken as a percentage of the number that should have been taken.

Statistical analyses

This study was adequately powered (80%) at the 5% significance level to detect a between groups difference in either of the bone turnover markers of at least one standard deviation. Effects of this size have previously been observed with calcium supplementation and are likely to be of clinical significance. Data were analysed on an intention-to-treat basis using a mixed models approach to repeated measures (Proc Mixed, SAS v 9.2, SAS Institute Inc).

The change from baseline was the dependent variable and the baseline value of the appropriate variable was included as a covariate (analysis of covariance (ANCOVA)). Significant main (time or treatment allocation) and interaction effects (time by treatment allocation) were further explored using the method of Tukey to construct honestly significant differences; however since these comparisons were pre-planned the pairwise P values were not adjusted for multiplicity. The areas under the ionised calcium, total calcium or phosphate over time functions (area under the curve (AUC)) were calculated using a trapezoidal method and provided the dependent variable as indicated. All tests were two-tailed and $P< 0.05$ was considered significant.

3.3 RESULTS

The baseline clinical and biochemical characteristics of participants are presented in Table 3.1. Over the 3 month study period, 85% of participants took 80% or more of their allocated treatment. Median compliance was not different between the groups ($p = 0.10$).

Table 3.1 Baseline clinical and biochemical characteristics of participants.

	Calcium citrate n = 18	Calcium carbonate n = 20	MCH A n = 20	MCH B n = 19	Control n = 20	Total N = 97
Clinical characteristics						
Age (years)	72 (6)	70 (4)	71 (5)	73 (6)	70 (3)	71 (5)
Weight (kg)	72.1 (10.5)	76.5 (14.9)	72.4 (15.3)	76.4 (12.1)	71.5 (9.2)	73.8 (12.6)
Height (m)	1.61 (0.06)	1.64 (0.06)	1.61 (0.07)	1.60 (0.06)	1.63 (0.06)	1.62 (0.06)
Dietary calcium (mg/day)	970 (380)	810 (320)	890 (420)	780 (400)	900 (500)	870 (410)
Biochemical characteristics						
25(OH)D (nmol/L)	80 (20)	71 (23)	69 (18)	70 (21)	68 (18)	71 (20)
Ionised calcium (mmol/L)	1.21 (0.03)	1.23 (0.06)	1.21 (0.03)	1.22 (0.06)	1.21 (0.03)	1.22 (0.04)
Total calcium (mmol/L)*	2.19 (0.07)	2.18 (0.08)	2.16 (0.07)	2.16 (0.09)	2.16 (0.09)	2.17 (0.09)
Phosphate (mmol/L)	1.06 (0.13)	1.10 (0.20)	1.12 (0.11)	1.14 (0.09)	1.11 (0.03)	1.11 (0.14)
PTH (pmol/L)	4.6 (1.2)	4.0 (1.3)	4.8 (2.2)	4.3 (1.2)	4.3 (1.2)	4.4 (1.5)
CTX (µg/L)	0.38 (0.11)	0.37 (0.15)	0.39 (0.15)	0.41 (0.18)	0.34 (0.14)	0.38 (0.15)
PINP (ug/L)	45.1 (14.0)	46.5 (19.1)	44.8 (15.2)	52.4 (28.7)	43.8 (15.3)	46.8 (19.3)

MCH = microcrystalline hydroxyapatite; 25(OH)D = 25-hydroxyvitamin D;PTH = parathyroid hormone; CTX = C-telopeptide; PINP = procollagen type-I N-terminal propeptide. *Total calcium corrected for albumin 40 g/l. Values are mean (SD).

Ionised calcium

Changes in serum ionised calcium over 8 hours are presented in Figure 3.2. Ionised calcium was significantly increased from baseline at all time-points between 2 and 8 hours in the calcium citrate (all time-points $p < 0.002$) and calcium carbonate (all $p < 0.0001$) groups and between 4 and 6 hours in the MCH A (all $p < 0.0001$) and MCH B (all $p < 0.046$) groups, and did not change from baseline in the control group. The change in ionised calcium was not significantly different at any time-point between the calcium citrate and carbonate groups, or between the MCH A and MCH B groups. To allow for comparison between the conventional calcium supplements and MCH, the results from these groups were pooled in subsequent analyses.

Changes in ionised calcium over 8 hours in the pooled groups are presented in Figure 3.3. Ionised calcium was significantly increased from baseline between 2 and 8 hours in the citrate-carbonate group (all time-points $p < 0.0001$) and between 4 and 8 hours in the MCH group (all $p < 0.01$). The maximal increase in ionised calcium in the citrate-carbonate group was 0.05 mmol/l, and in the MCH group was 0.03 mmol/l, and was reached at 4 hours. Ionised calcium remained elevated in the citrate-carbonate group by 0.03 mmol/l, and in the MCH group by 0.02 mmol/l, at 8 hours. There were significant differences in the change in ionised calcium between the groups, as presented in Figure 3.3. A total of 15 participants in the citrate-carbonate group, 4 in the MCH group, and none in the control group had an ionised calcium measurement above the normal reference range (greater than 1.30 mmol/l) at one or more time-points over 8 hours. The AUCs for ionised calcium are presented in Figure 3.4. The AUC for ionised calcium was greater in the citrate-carbonate group than in the MCH or control groups.

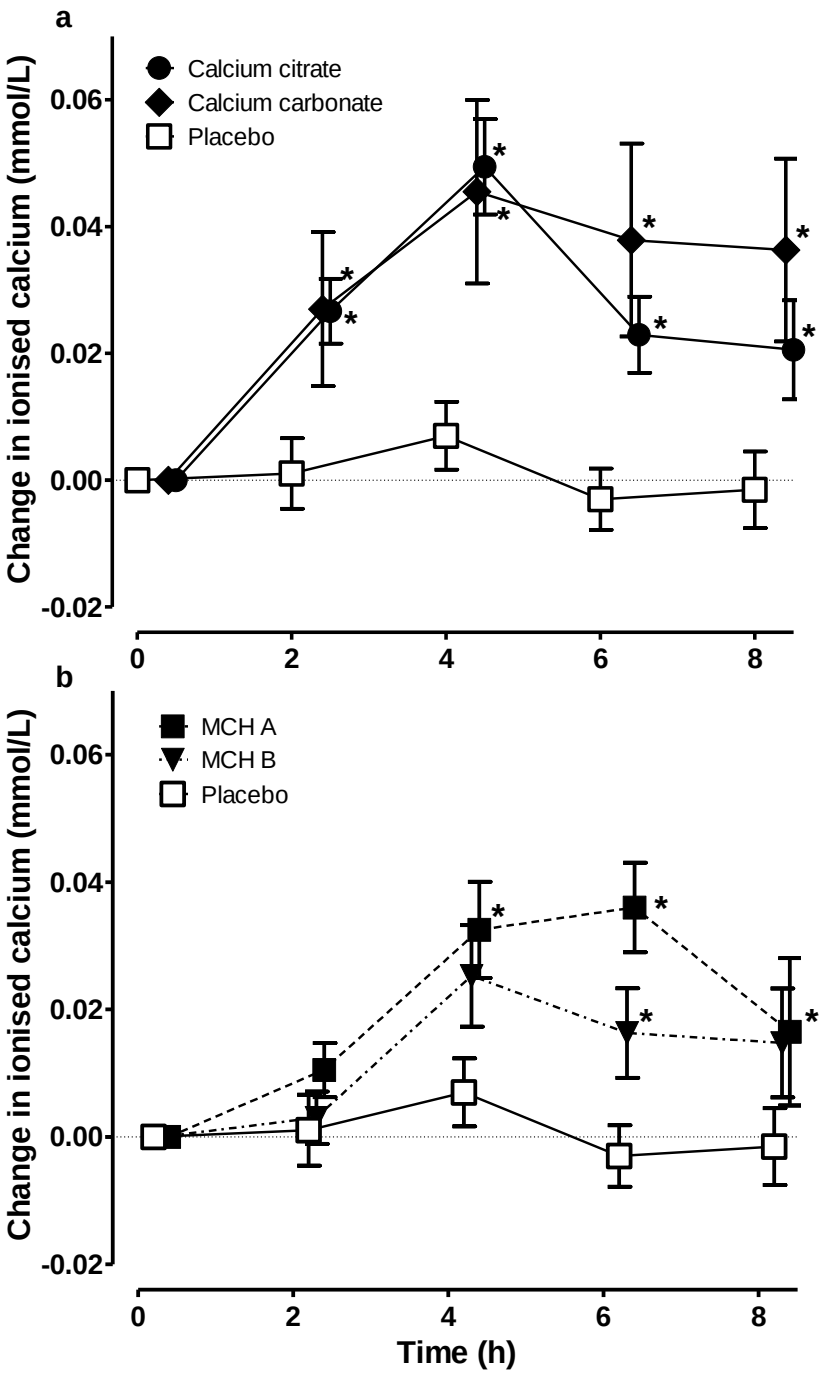

Figure 3.2 Change in serum ionised calcium over 8 hours after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 20), MCH B (n = 19), or a placebo containing no calcium (control; n = 20). Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and MCH B in graph b. Changes after placebo are shown in both graphs. There was a significant treatment x time interaction for the change in ionised calcium among the five groups (ANCOVA, p = 0.004). * Significantly different from the control, p <0.05. Values are mean ± SEM.

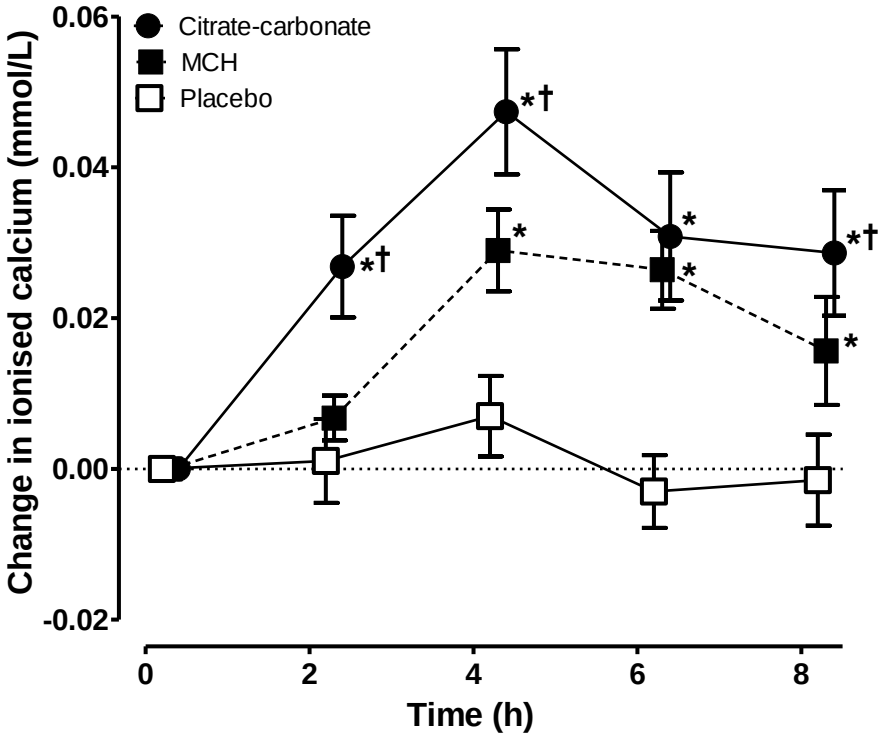

Figure 3.3 Change in serum ionised calcium over 8 hours after the ingestion of 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20). There was a significant treatment x time interaction for the change in ionised calcium among the three groups (ANCOVA, p = 0.0008). * Significantly different from control, p <0.05; † significantly different from MCH, p <0.05. Values are mean ± SEM.

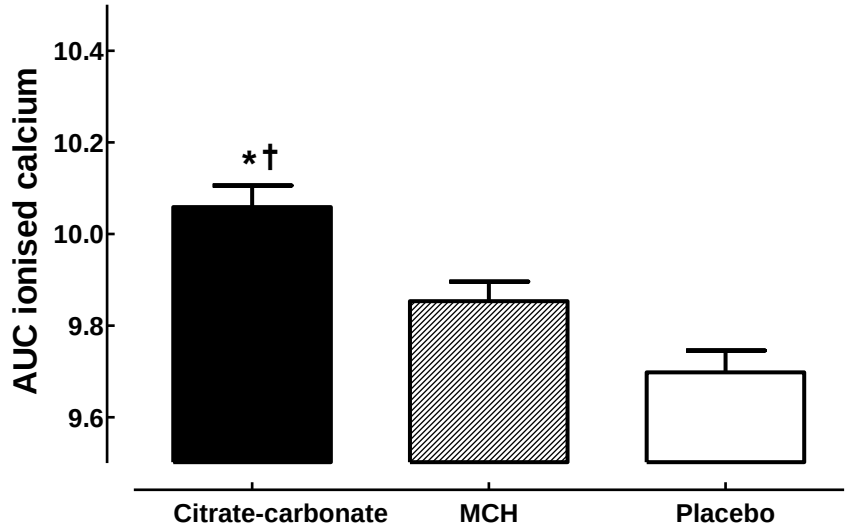

Figure 3.4 Area under the curve (AUC) of the change in serum ionised calcium over 8 hours after 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20). * Significantly different from control, p<0.05; † significantly different from MCH, p <0.05. Values are mean ± SEM.

Changes in ionised calcium in the eight participants allocated to calcium citrate who repeated the 8 hour session at 3 months are presented in Figure 3.5. At the baseline visit, ionised calcium was significantly increased from baseline between 2 and 8 hours after the ingestion of calcium citrate ($p < 0.002$). At the 3 month visit, ionised calcium was significantly increased from the fasting concentration between 2 and 6 hours after the ingestion of calcium citrate ($p < 0.01$). The maximal increase in ionised calcium at the baseline visit was 0.05 mmol/l, and was reached at 4 hours. Ionised calcium remained elevated at the baseline visit by 0.02 mmol/l at 8 hours. The maximal increase in ionised calcium at the 3 month visit was 0.03 mmol/l, and was reached at 4 hours. There were no differences in the change in ionised calcium over 8 hours between the baseline and 3 month visits (Figure 2.5).

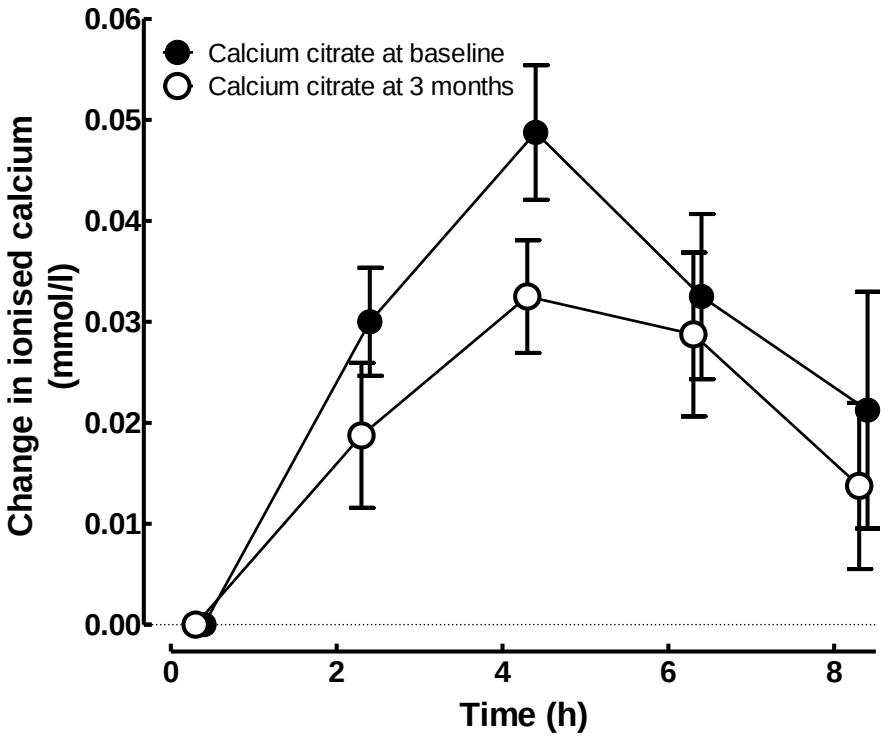

Figure 3.5 Change in ionised calcium over 8 hours after the ingestion of 1000 mg of calcium as citrate at the baseline visit or at the 3 month visit (n = 8). There was no interaction between the visit and time ($p = 0.62$). Values are mean ± SEM.

Total calcium

Changes in serum total calcium over 8 hours are presented in Figure 3.6. Total calcium was significantly increased from baseline at all time-points between 2 and 8 hours in the calcium citrate (all time-points $p < 0.004$), calcium carbonate (all $p < 0.0001$) and MCH A groups (all $p < 0.002$), and between 2 and 6 hours in the MCH B group (all $p < 0.03$). Total calcium was lower than baseline at 8 hours in the control group ($p = 0.009$). The change in total calcium was not different at any time point between the calcium citrate and carbonate groups, or between the MCH A and MCH B groups. To allow for comparison between the conventional calcium supplements and MCH, the results from these groups were pooled.

Changes in total calcium in the pooled groups are presented in Figure 3.7. Total calcium was significantly increased from baseline between 2 and 8 hours in the citrate-carbonate group (all time-points $p < 0.0001$) and the MCH group (all $p < 0.001$). The maximal increase in total calcium in the citrate-carbonate group was 0.09 mmol/l, and in the MCH group was 0.07 mmol/l, and was reached at 4 hours. Total calcium remained elevated in the citrate-carbonate group by 0.04 mmol/l, and in the MCH group by 0.03 mmol/l, at 8 hours. There were significant differences between the groups in the change in total calcium, as presented in Figure 3.7. One participant in the citrate-carbonate group and no participants in the MCH or control groups had a total calcium measurement above the normal reference range (greater than 2.55 mmol/l) at one or more time-points over 8 hours. The AUCs for total calcium are presented in Figure 3.8. The AUCs for total calcium were greater in the citrate-carbonate and MCH groups than the control group.

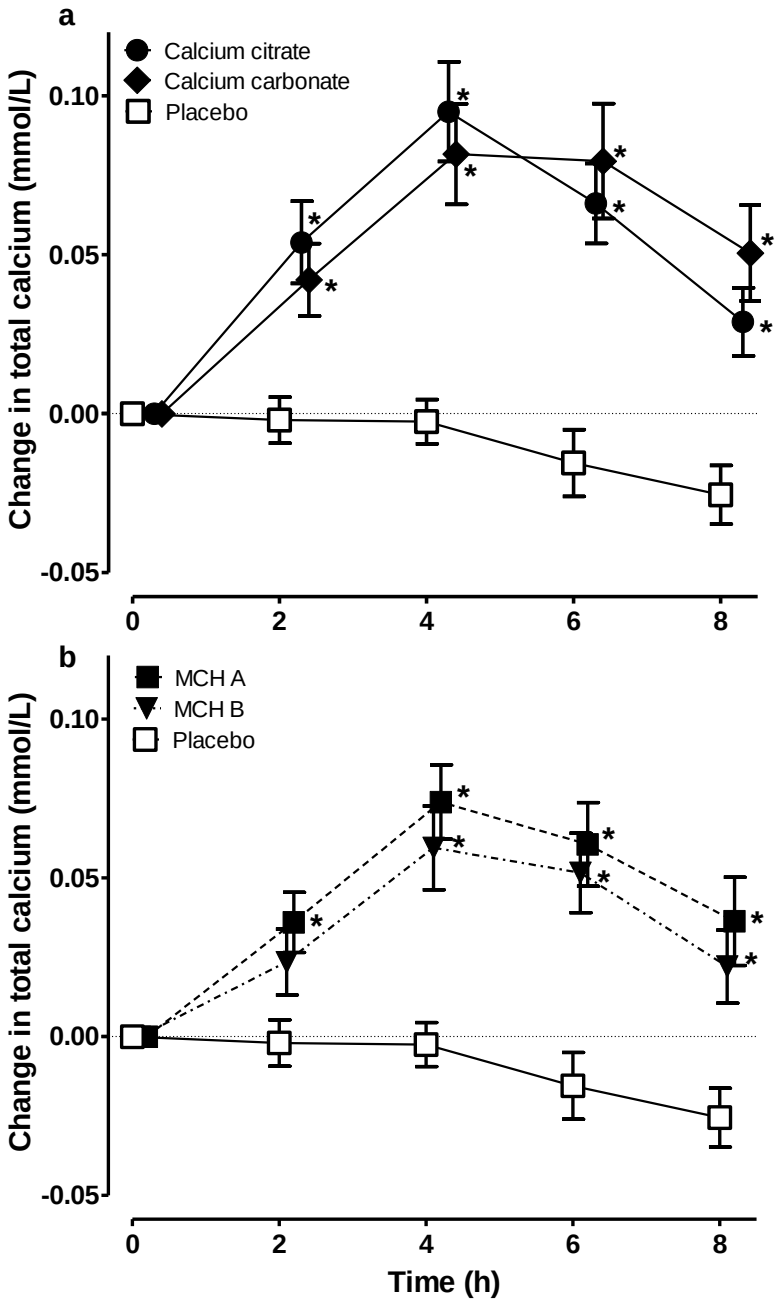

Figure 3.6 Change in serum total calcium over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 20) or MCH B (n = 19), or a placebo containing no calcium (control; n = 20). Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and B in graph b. Changes after placebo are shown in both graphs. There was a significant treatment x time interaction for the change in total calcium among the five groups (ANCOVA, $p < 0.0001$). * Significantly different from control, $p < 0.05$. Values are mean ± SEM.

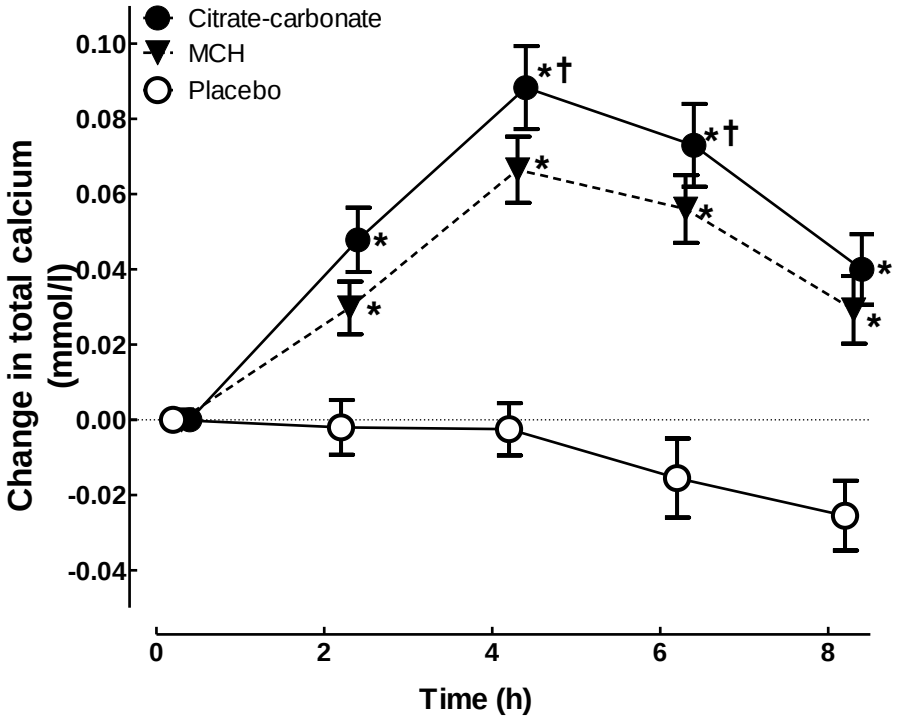

Figure 3.7 Change in serum total calcium over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20). There was a significant treatment by time interaction for the change in total calcium among the three groups (ANCOVA, $p < 0.0001$). * Significantly different from control, $p < 0.05$; † significantly different from MCH, $p < 0.05$. Values are mean ± SEM.

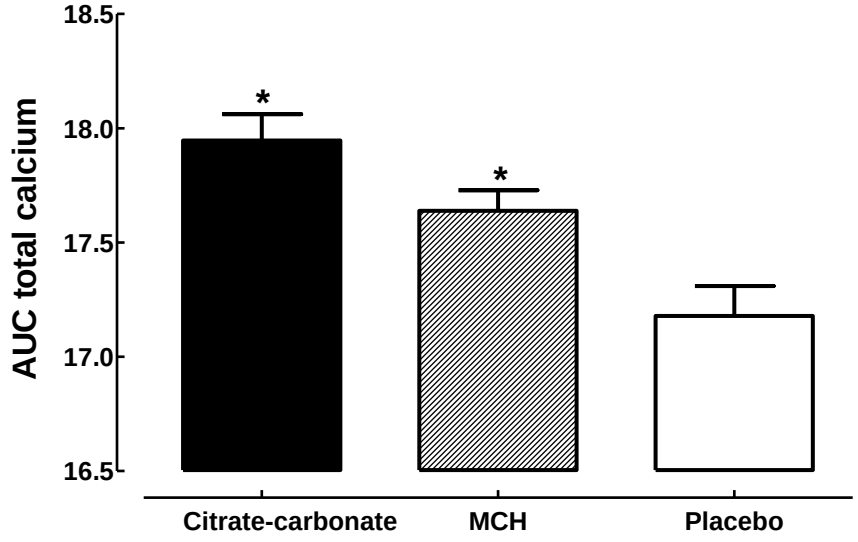

Figure 3.8 Area under the curve (AUC) of the change in serum total calcium over 8 hours after 1000 mg of calcium as citrate or carbonate (n = 38), MCH (n = 39), or a placebo containing no calcium (control; n = 20). * Significantly different from control, $p < 0.05$. Values are mean ± SEM.

Changes in total calcium in the eight participants allocated to calcium citrate who repeated the 8 hour session at 3 months are presented in Figure 3.9. At the baseline visit total calcium was significantly increased from baseline between 2 and 6 hours after the ingestion of calcium citrate ($p < 0.009$). At the 3 month visit, total calcium was significantly increased from the fasting concentration between 2 and 8 hours after the ingestion of calcium citrate ($p < 0.008$). The maximal increase in total calcium after calcium citrate at baseline was 0.08 mmol/l, and was reached at 4 hours. The maximal increase in total calcium after calcium citrate at 3 months was 0.11 mmol/l, and was reached at 4 hours. Total calcium remained elevated at the 3 month visit by 0.06 mmol/l at 8 hours. There were no differences in the change in total calcium between the baseline and 3 month visits (Figure 3.9).

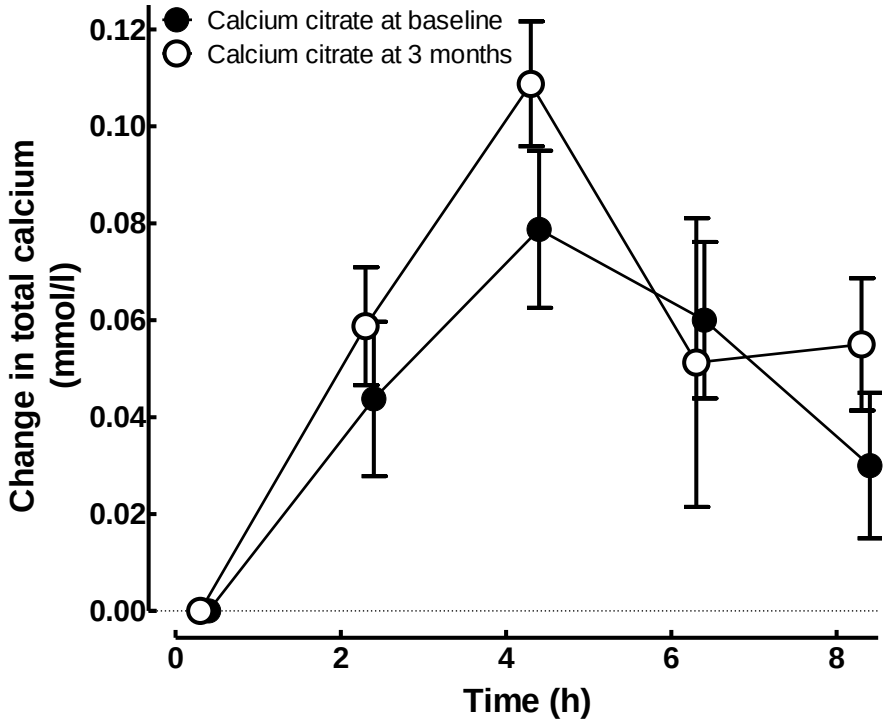

Figure 3.9 Change in total calcium over 8 hours after the ingestion of 1000 mg of calcium as citrate, taken at the baseline visit or the 3 month visit (n = 8). There was no interaction between the visit and time (ANCOVA, p = 0.61). Values are mean ± SEM.

Phosphate

Changes in serum phosphate over 8 hours are presented in Figure 3.10. Phosphate was lower than baseline at 2 hours in all groups (all $p < 0.0001$). Between 4 and 8 hours phosphate was not different from baseline in the calcium citrate and calcium carbonate groups, and was increased from baseline in the MCH A (all $p < 0.003$) and MCH B (all $p < 0.03$) groups. Between 6 and 8 hours phosphate was lower than baseline in the control group (all $p < 0.003$). The change in phosphate over 8 hours was not significantly different at any time-point between the calcium citrate and carbonate groups or between the MCH A and MCH B groups. As previously, the results from these groups were pooled.

Changes in phosphate in the pooled groups are presented in Figure 3.11. Phosphate was lower than baseline at 2 hours by 0.10 to 0.13 mmol/l in all groups (all $p < 0.0001$). In the citrate-carbonate group, phosphate was not different from baseline between 4 and 8 hours. In the MCH group, phosphate was increased from baseline between 4 and 8 hours (all $p < 0.0001$). The maximal increase in phosphate in the MCH group was 0.10 mmol/l, and was reached at 4 hours. Phosphate remained elevated from baseline in the MCH group by 0.05 mmol/l at 8 hours. In the control group, phosphate was not different from baseline at 4 hours, and was lower than baseline between 6 and 8 hours (all $p < 0.003$). The maximal reduction in phosphate in the control group was 0.06 mmol/l, and was reached at 8 hours. There were significant differences between the groups in the change in phosphate, as presented in Figure 3.11. The AUCs for phosphate are presented in Figure 3.12. The AUC for phosphate was greater in the MCH group than the citrate-carbonate and control groups.

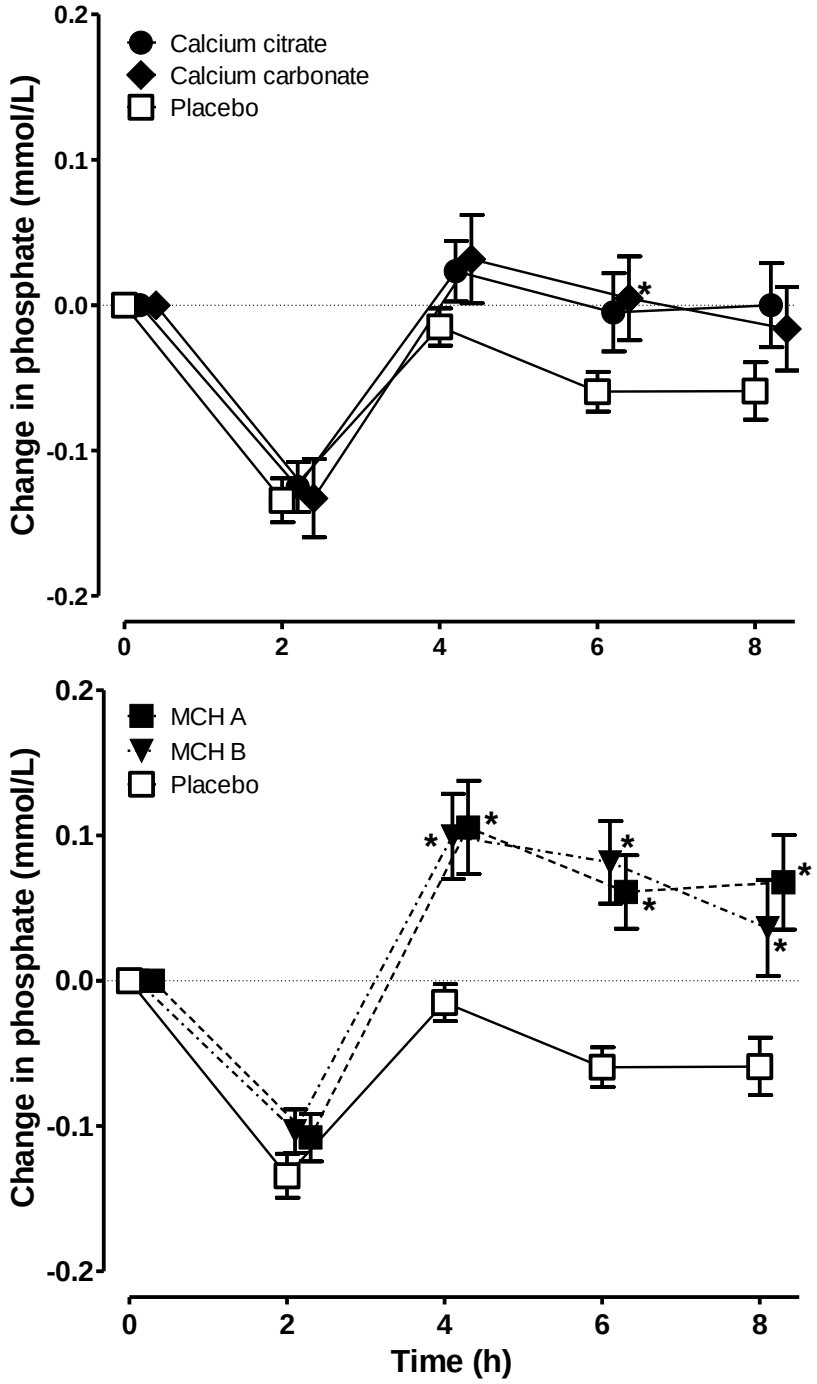

Figure 3.10 Change in serum phosphate over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 20), MCH B (n = 19), or a placebo containing no calcium (control; n = 20). Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and B in graph b. Changes after placebo are shown in both graphs. There was a significant treatment x time interaction for the change in phosphate among the five groups (ANCOVA, p = 0.0008). * Significantly different from control, p <0.05. Values are mean ± SEM.

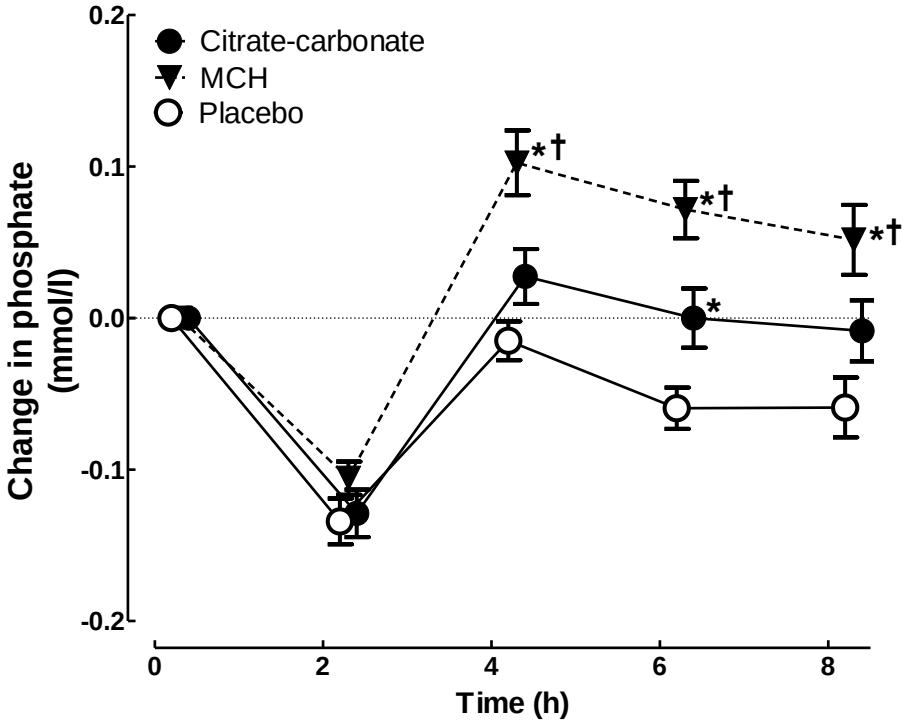

Figure 3.11 Change in serum phosphate over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20). There was a significant treatment x time interaction for the change in phosphate among the three groups (ANCOVA, $p < 0.0001$). * Significantly different from control, $p < 0.05$; † significantly different from calcium citrate or carbonate, $p < 0.05$. Values are mean ± SEM.

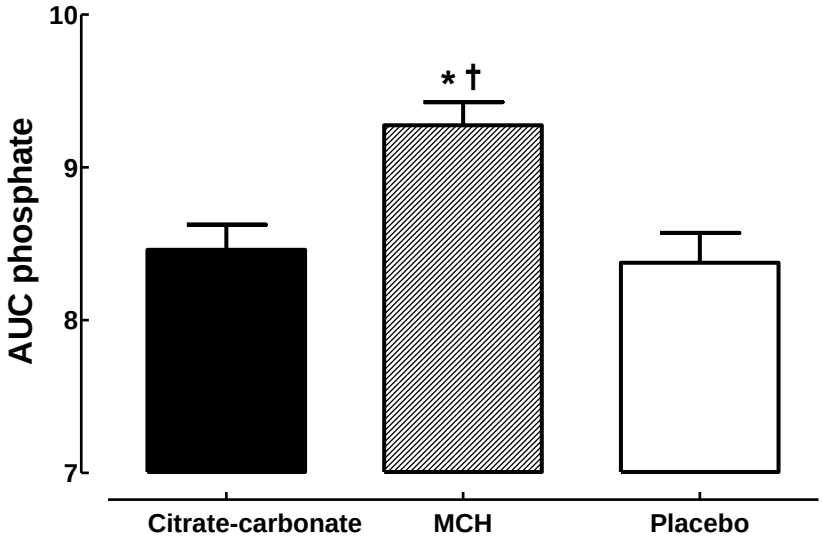

Figure 3.12 Area under the curve (AUC) of the change in serum phosphate over 8 hours after 1000 mg of calcium as citrate or carbonate (n = 38), MCH (n = 39), or a placebo containing no calcium (control; n = 20). * Significantly different from control, $p < 0.05$; † significantly different from calcium citrate or carbonate, $p < 0.05$. Values are mean ± SEM.

Calcium-phosphate product

The time-course of the change in calcium-phosphate product was examined in the pooled treatment groups (Figure 3.13). The calcium-phosphate product was lower than baseline at 2 hours by 12 to 13 $mmol^2/l^2$ in the citrate-carbonate and MCH groups (all $p > 0.0001$), and by 17 $mmol^2/l^2$ in the control group ($p < 0.0001$). In the citrate-carbonate group, the calcium-phosphate product was increased from baseline at 4 hours by 0.09 $mmol^2/l^2$ ($p < 0.001$). In the MCH group, the calcium-phosphate product was increased from baseline between 4 and 8 hours (all $p < 0.0001$). The maximal increase in the calcium-phosphate product in the MCH group was 0.16 $mmol^2/l^2$, and was reached 4 hours. The calcium-phosphate product remained elevated in the MCH group by 0.08 $mmol^2/l^2$ at 8 hours. In the control group, the calcium-phosphate product was lower than baseline at 6 and 8 hours (all $p < 0.007$). The maximal reduction in calcium-phosphate product in the control group was 0.07 $mmol^2/l^2$ ($p = 0.006$), and was reached at 8 hours. There were significant differences between the groups in the change in the calcium-phosphate product, as presented in Figure 3.13.

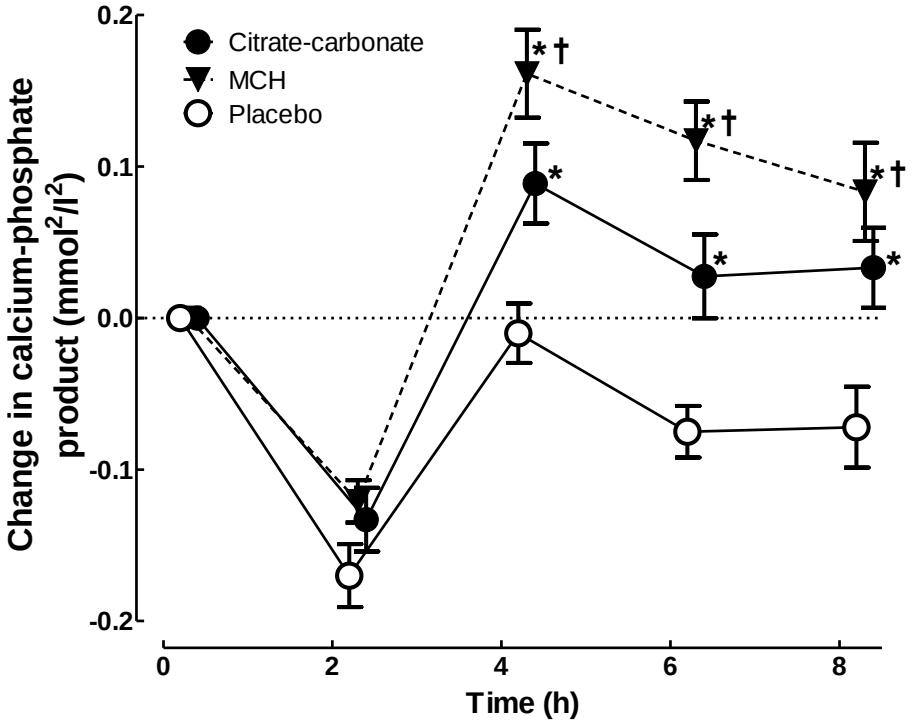

Figure 3.13 Change in serum calcium-phosphate product over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20). There was a significant treatment x time interaction for the change in calcium-phosphate product ($p < 0.0001$). * Significantly different from control, $p < 0.05$; † significantly different from calcium citrate or carbonate, $p < 0.05$. Values are mean ± SEM.

Parathyroid hormone

Percentage changes in PTH are presented in Figure 3.14. PTH was lower than baseline between 2 and 8 hours in the calcium carbonate (all time-points $p < 0.0004$), calcium citrate (all $p < 0.047$) and MCH B groups (all $p < 0.04$), between 2 and 6 hours in the MCH A group (all $p < 0.0001$), and at 2 and 6 hours in the control group (all $p < 0.03$). After 3 months, PTH was lower than baseline in the calcium citrate ($p = 0.05$) and MCH A ($p = 0.03$) groups and nonsignificantly lower in the carbonate group ($p = 0.07$), but not different from baseline in the MCH B or control groups. The percentage change in PTH was not different between the calcium citrate and carbonate groups, or between the MCH A and MCH B groups. As previously, the results from these groups were pooled.

Percentage changes in PTH in the pooled groups are presented in Figure 3.15. In the control group PTH was lower than baseline at 2 hours by 19% ($p < 0.0001$) and at 6 hours by 7% ($p = 0.02$), and was not different from baseline at any other time-point. PTH was lower than baseline between 2 and 8 hours in the citrate-carbonate (all $p<0.0001$) and MCH (all $p < 0.006$) groups. The maximal reduction in PTH in the citrate-carbonate group was 28%, and was reached at 4 hours. PTH remained lower than baseline in the citrate-carbonate group by 9% at 8 hours. The maximal reduction in PTH in the MCH group was 22%, and was reached at 6 hours. PTH remained lower than baseline in the MCH group by 6% at 8 hours. After 3 months PTH was lower than baseline values by 4% in the citrate-carbonate group ($p = 0.009$), but not different from baseline in the MCH or control groups. The changes in PTH were significantly different between the groups, as presented in Figure 3.15.

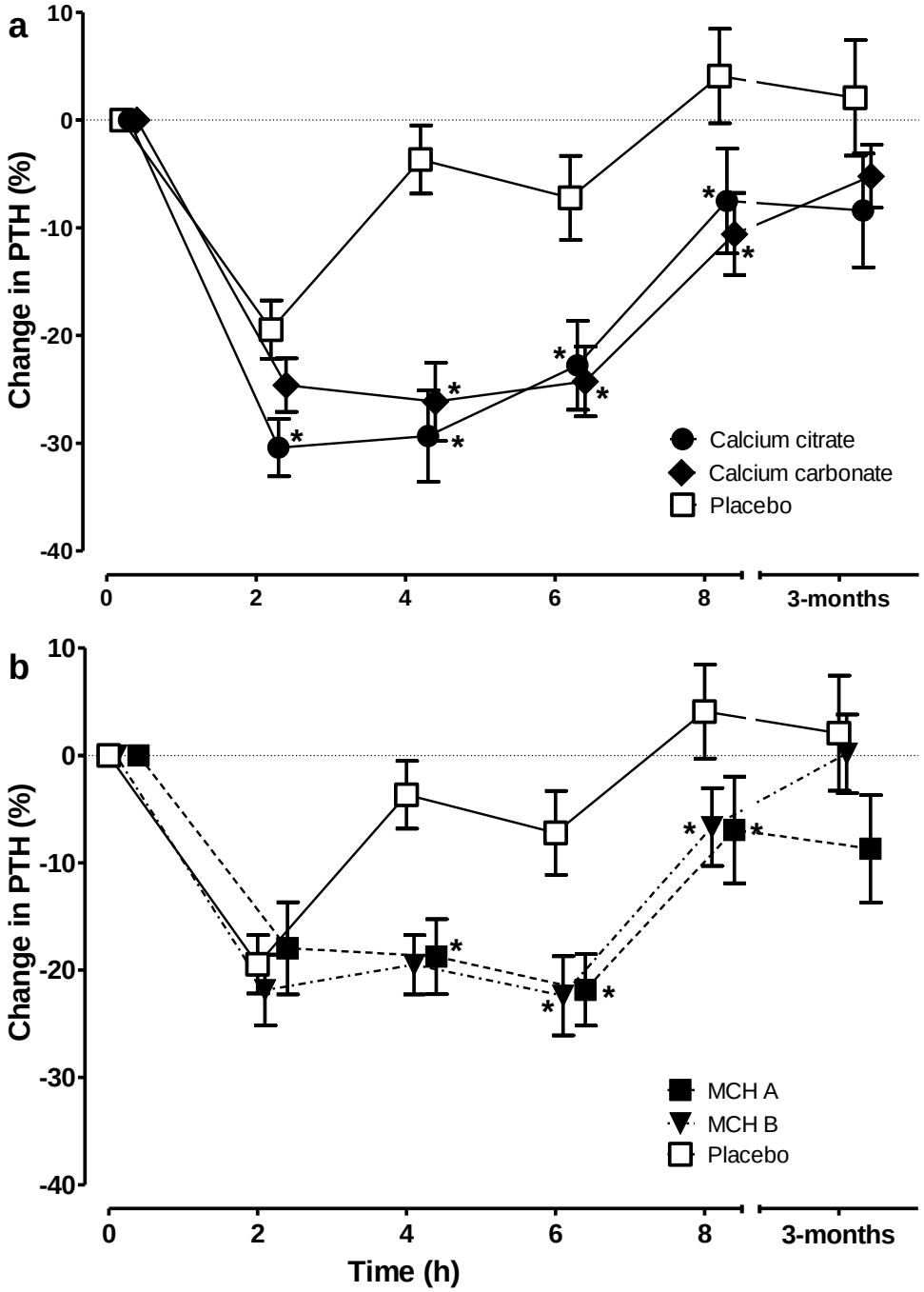

Figure 3.14 Percentage change in serum PTH over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 20), MCH B (n = 19), or a placebo containing no calcium (control; n = 20), and after 3-months of supplementation. Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and B in graph b. Changes after placebo are shown in both graphs. There was a significant treatment x time interaction for the change in PTH among the five groups (ANCOVA, p = 0.041). * Significantly different from control, p <0.05. Values are mean ± SEM.

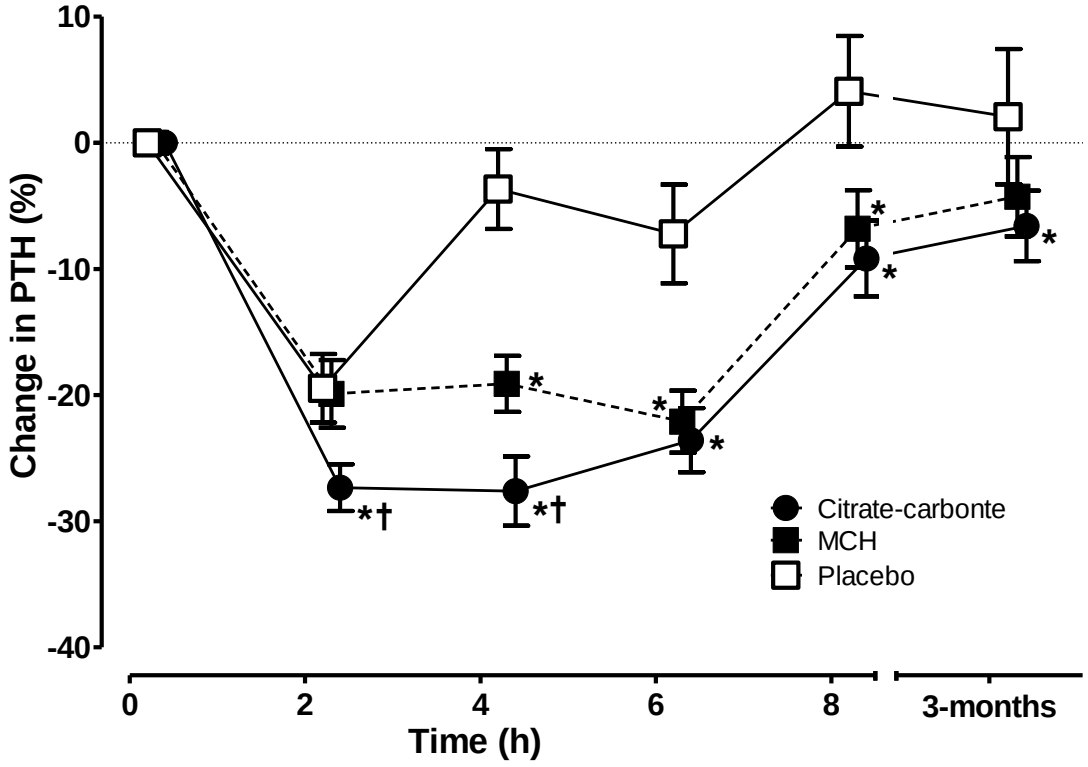

Figure 3.15 Percentage change in serum PTH over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20), and after 3-months of supplementation. There was a significant treatment x time interaction for the change in PTH among the three groups (ANCOVA, p = 0.004). * Significantly different from control, p <0.05; † significantly different from MCH, p <0.05. Values are mean ± SEM.

CTX

Percentage changes in CTX are presented in Figure 3.16. CTX was lower than baseline at all time-points between 2 and 8 hours in the calcium citrate, calcium carbonate, MCH A, MCH B and control groups (all time-points $p < 0.0001$). At 3 months, CTX was lower than baseline in the calcium citrate, calcium carbonate, MCH A and MCH B groups ($p < 0.0001$), and nonsignificantly lower than baseline in the control group ($p = 0.06$). The percentage change in CTX was not different between the calcium citrate and carbonate groups or between the MCH A and MCH B groups. As previously, the results from these groups were pooled.

Percentage changes in CTX in the pooled groups are presented in Figure 3.17. CTX was lower than baseline at all time-points between 2 and 8 hours in the control, citrate-carbonate and MCH groups (all time-points $p < 0.0001$). The maximal reduction in CTX in the control group was 43%, and was reached at 6 hours. CTX remained lower than baseline in the control group by 36% at 8 hours. The maximal reduction in CTX in the citrate-carbonate and MCH groups was 57 - 59%, and was reached at 6 hours. CTX remained lower than baseline in the citrate-carbonate and MCH groups by 52 - 53% at 8 hours. After 3 months, CTX was nonsignificantly lower than baseline by 5% in the control group ($p = 0.06$) was lower than baseline by 30 - 34% in the citrate-carbonate and MCH groups (all $p < 0.0001$). There were significant differences between the groups in the change in CTX, as presented in Figure 3.17.

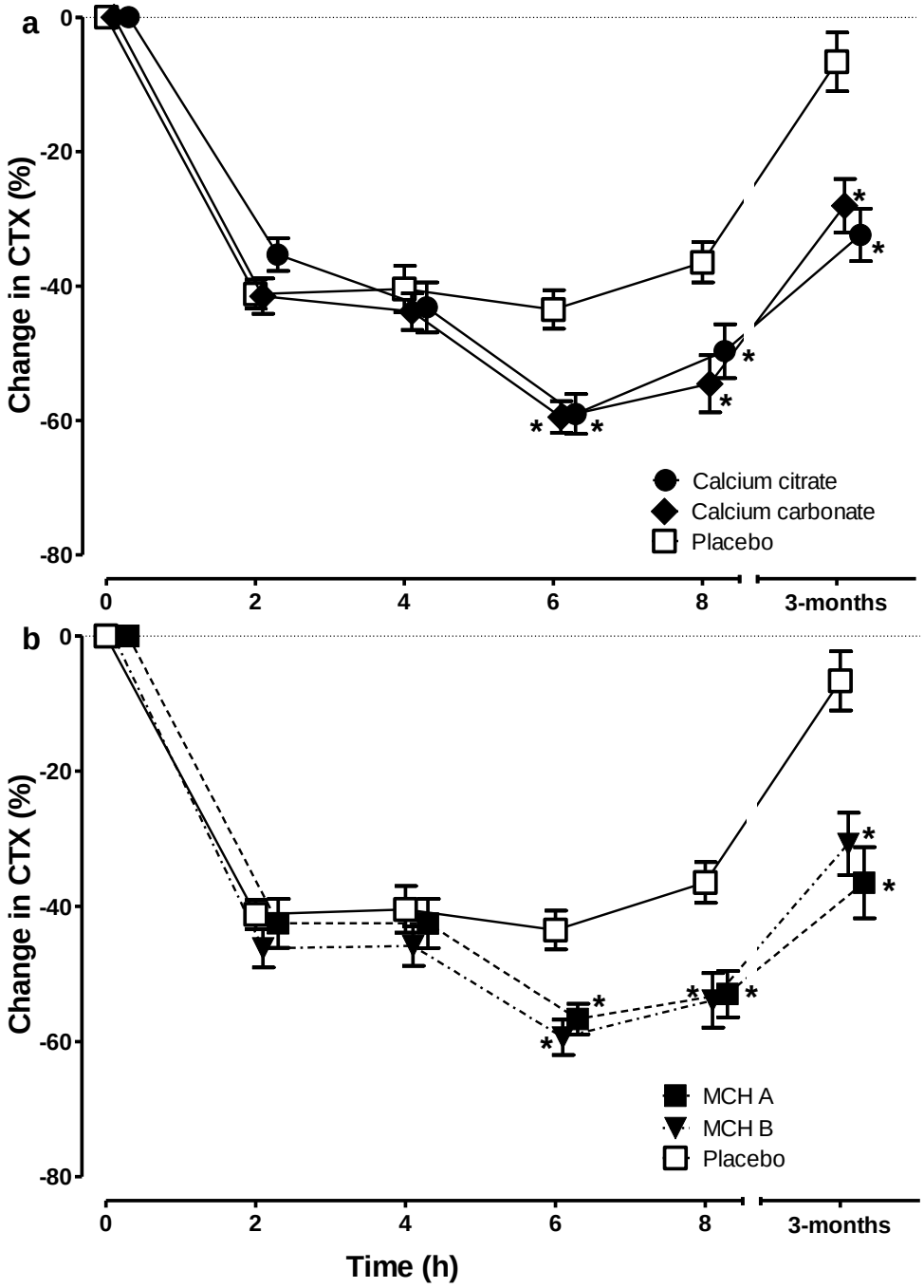

Figure 3.16 Percentage change in serum CTX over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 20), MCH B (n = 19), or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and B in graph b. Changes after placebo are shown in both graphs. There was a significant treatment x time interaction for the change in CTX among the five groups (ANCOVA, p = 0.0006). * Significantly different from control, p <0.05. Values are mean ± SEM.

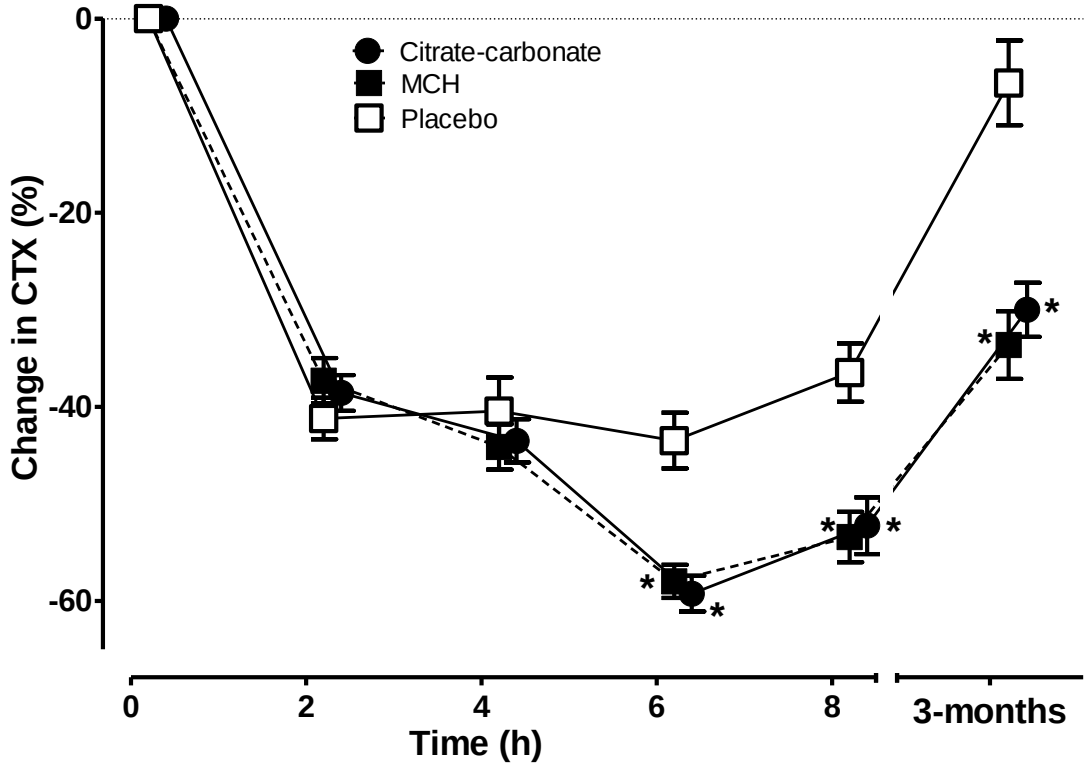

Figure 3.17 Percentage change in serum CTX over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. There was a significant treatment x time interaction for the change in CTX among the three groups (ANCOVA, p = 0.0002). * Significantly different from control, p <0.05. Values are mean ± SEM.

PINP

Percentage changes in PINP are presented in Figure 3.18. PINP was increased from baseline at 8 hours in the calcium citrate ($p = 0.002$), calcium carbonate ($p = 0.002$), MCH B ($p = 0.02$) and control ($p = 0.04$) groups, and was not different from baseline at 8 hours in the MCH A group ($p = 0.13$). After 3 months, PINP was lower than baseline in the calcium citrate, calcium carbonate, MCH A and MCH B groups (all $p < 0.0001$), and not different from baseline in the control group. Changes in PINP were not different between the calcium citrate and carbonate groups, or between the MCH A and MCH B groups. As previously, the results from these groups were pooled.

Percentage changes in PINP in the pooled groups are presented in Figure 3.19. PINP was significantly increased from baseline at 8 hours by 6 - 9% in the control ($p = 0.03$), citrate-carbonate ($p < 0.0001$), and MCH ($p = 0.006$) groups. After 3 months, PINP was lower than baseline by 14 - 19% in the citrate-carbonate and MCH groups (all $p < 0.0001$), and not different from baseline in the control group. There were significant differences between the groups in the change in PINP at 3 months, as presented in Figure 3.19.

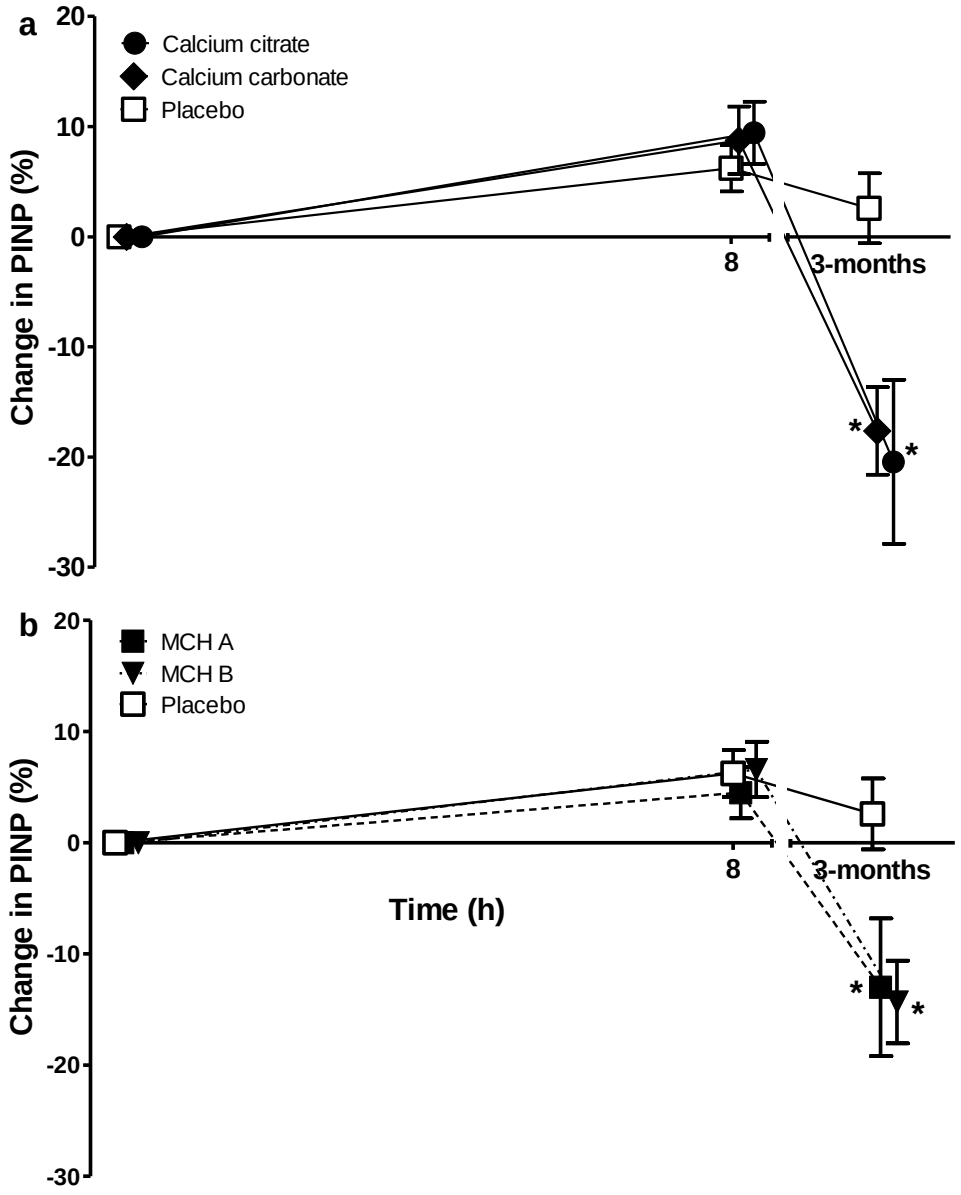

Figure 3.18 Percentage change in serum PINP in postmenopausal women 8 hours after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 20), MCH B (n = 19), or a placebo containing no calcium (control; n = 20), and after 3-months of supplementation. Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and MCH B in graph b. Changes after placebo are shown in both graphs. There was a significant treatment x time interaction for the change in PINP among the five groups ($p = 0.002$). * Significantly different from control, $p < 0.05$. Values are mean ± SEM.

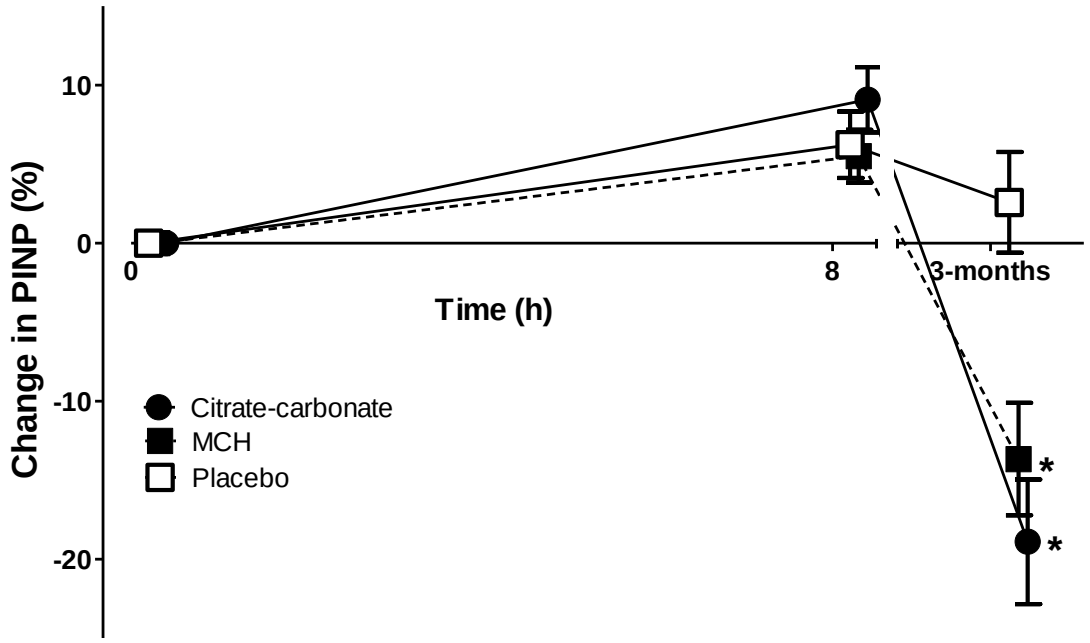

Figure 3.19 Percentage change in serum PINP in postmenopausal women 8 hours after the ingestion of 1000 mg of calcium as citrate or carbonate (n = 38) or MCH (n = 39), or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. There was a significant treatment x time interaction for the change in PINP among the three groups ($p < 0.0001$). * Significantly different from control, $p < 0.05$. Values are mean ± SEM.

3.4 DISCUSSION

In the present study, 1000 mg of calcium as citrate, carbonate or MCH resulted in an acute elevation in serum calcium and suppression of bone resorption; and after 3 months of supplementation, a global reduction in bone turnover (reflected in both CTX and PINP levels). Compared with the conventional calcium supplements, MCH resulted in a smaller acute increase in ionised calcium, but suppressed bone turnover comparably.

Ionised and total calcium remained elevated above baseline and the control group 8 hours after the ingestion of 1000 mg of calcium. Previous studies have demonstrated elevations in serum calcium up to 6 hours after an oral dose [68, 140, 278], but few have examined effects beyond this time-point. In a cross-over trial of postmenopausal women, serum calcium was significantly greater than control 3 to 12 hours after calcium carbonate and 1 to 9 hours after calcium citrate [69]. Similarly, in a cross-over trial of postmenopausal women, an evening dose of calcium carbonate or citrate raised serum calcium for at least 12 hours [279]. Furthermore, in the present study, the serum calcium excursion was not different when calcium citrate was taken at baseline, or after 3 months of supplementation, suggesting these effects are not diminished with long-term use. In longitudinal studies [182] differences in serum calcium of the magnitude observed here are associated with a 17% increase in cardiovascular risk, very similar to that found in trials of calcium supplementation [29].

Few studies have compared the acute biochemical effects of MCH with other forms of calcium. Consistent with the findings of the present study, these studies have demonstrated smaller increases in serum calcium and/or reductions in PTH following the ingestion of MCH compared with other calcium salts [68, 71, 280]. The smaller rise in serum calcium following MCH may have been due to a lower bioavailability of calcium. This was suggested by the smaller AUC of ionised calcium following MCH compared with citrate-carbonate. Alternatively, calcium may have been more slowly absorbed from MCH because of slow dissolution of the hydroxyapatite crystal in the gastrointestinal tract, and the need for hydrolysis of the protein matrix. Furthermore, in contrast to ionised calcium, the AUC of total calcium was not different between the citrate-carbonate and MCH groups. Increased serum phosphate might explain the smaller effect of MCH on ionised calcium, since phosphate complexes with calcium.

There were no differences in any parameter between the citrate and carbonate groups, or between the two MCH groups. Calcium citrate is more soluble than carbonate, and has been shown to be more absorbable and/or have a greater calcaemic effect [279, 281, 282], although this has not been consistent [68-70]. Solubility has a limited role in determining calcium absorption, when calcium is administered with a meal [283, 284]. Differences between the carbonate and citrate salts in some trials might reflect differences in the physical form in which they were administered (for example powder versus tablet) [285]. In the present study all preparations were administered as identical encapsulated powders. Similarly, the more finely processed MCH preparation would be expected to be more rapidly absorbed than the less finely processed preparation, due to the greater surface area of the smaller particles, but this was not the observed outcome.

PTH exhibits a marked diurnal rhythm [286] and is affected by feeding [287], as are markers of bone resorption, such as CTX [288-292]. Accordingly, there were significant changes in PTH and CTX in the control group over 8 hours; these changes were very similar to those previously reported for PTH [286] and CTX [290]. The citrate-carbonate and MCH preparations further reduced CTX over 8 hours compared with the control. Markers of bone formation, such as PINP, exhibit a diurnal rhythm similar to those of bone resorption, however one that is less pronounced [293]. PINP was only measured at 8 hours in the present study, and was elevated in all groups at this time. After 3 months, bone turnover was significantly lower in the citrate-carbonate and MCH groups, reflected by reductions in both CTX and PINP concentrations. Lowered bone turnover is a well-established effect of calcium supplementation [62, 71, 137, 294].

Previous studies have compared the acute effects of different calcium preparations on serum calcium [68, 69, 71, 282], under the premise that larger increases in ionised calcium will translate into greater suppression of bone turnover. Few studies have, however, examined whether these acute differences in serum calcium translate into long-term differences in bone turnover. Despite the smaller increase in ionised calcium following the ingestion of MCH, the effects on CTX and PINP were the same, both acutely and after 3 months of supplementation. Consistently, trials of greater duration have demonstrated that MCH is as effective as calcium carbonate in slowing bone loss [295-297]. Collectively, these findings suggest that the acute effects of calcium preparations on serum calcium might be disassociated from their longer-term effects on bone turnover.

There was a reduction in serum phosphate at 2 hours in all groups. This may have been an effect of the breakfast, since carbohydrate after a fast promotes movement of phosphate into cells [298]. At 4 hours, phosphate was elevated in the MCH group compared with the control and citrate-carbonate groups. Participants allocated to MCH would have received an additional 0.5 g of phosphate (based on a calcium to phosphate ratio in bone of 1.5) compared with the other groups. Higher serum phosphate and calcium-phosphate product have been associated with increased cardiovascular disease risk in the general population [183, 299-301], suggesting MCH might not provide a safer form of supplemental calcium. Phosphate was slightly higher in the citrate-carbonate group relative to the control. This might be explained by the suppression of PTH, since PTH is phosphaturic.

In summary, the ingestion of conventional calcium citrate and carbonate supplements elevated serum calcium for at least 8 hours, and this elevation was not diminished with continuous use. Compared with the conventional calcium supplements, MCH resulted in a smaller increase in ionised calcium and a marginally smaller reduction in PTH, but suppressed bone turnover comparably. Raised serum phosphate and calcium-phosphate product after MCH, however, suggest it may not provide a safer form of supplemental calcium. For people unable to consume adequate calcium through diet alone, future research could aim to identify forms of supplemental calcium which release calcium into the blood slowly. The findings of the present study suggest calcium preparations with smaller effects on serum calcium may still retain comparable efficacy in suppressing bone turnover.

CHAPTER 4: ACUTE AND 3-MONTH EFFECTS OF CALCIUM SUPPLEMENTS ON BLOOD PRESSURE, BLOOD COAGULATION AND REGULATORS OF VASCULAR CALCIFICATION: A RANDOMISED CONTROLLED TRIAL

4.1 INTRODUCTION

Calcium supplementation has been associated with increased cardiovascular risk [29]. In Chapter 3, I demonstrated that serum calcium is elevated for up to 8 hours following the ingestion of a calcium supplement, and that is not diminished after 3 months of continuous use. It is possible that this elevation in serum calcium underlies the increase in cardiovascular risk associated with calcium supplementation. In observational studies, high-normal serum calcium concentrations have been associated with increased cardiovascular risk [179-182].

Calcium is involved in a diverse range of biological processes, and calcium supplementation could influence cardiovascular risk in several ways. Long-term calcium supplementation modestly reduces blood pressure [150, 153, 156, 159], while an infusion of calcium has a hypertensive effect [194, 200, 202, 203]. Calcium is also essential for blood coagulation, and platelets express the calcium-sensing receptor [209, 210]. The use of calcium as a phosphate binder in patients with CKD is associated with vascular calcification [160-162], as are increased serum calcium concentrations in the general population [223, 302]. Therefore, there exist several mechanisms by which calcium supplementation and the associated changes in serum calcium might increase cardiovascular risk, however there is little evidence to document their potential role. As a secondary aim in the RCT described in Chapter 3, I examined the effects of calcium supplements on changes in blood pressure, blood coagulation and regulators of vascular calcification. These results are presented here.

4.2 METHODS

Recruitment, participants and the design of the study are presented in Chapter 3. The flow of participants through the study is presented in Figure 3.1.

Measurements

Blood pressure

Blood pressure was measured on participants from all groups. One participant declined to have blood pressure measured, so results are available for 96 participants. Blood pressure was measured using a Dinamap automatic monitor (Johnson & Johnson, Tampa, FL) at baseline and 2, 4, 6, and 8 hours after the first dose of allocated treatment was ingested. After 3 months of continuous supplementation, fasting blood pressure was measured the morning after an evening dosing. At each time-point, three recordings were made 3 minutes apart, as programmed automatically by the device. Across the cohort, the first recording was found to be significantly higher than the following two recordings, so the mean of second and third recordings was used in the analyses.

Blood coagulation

Blood coagulation was measured by TEG on participants allocated to calcium citrate or placebo. The calcium citrate group was selected for these measurements as calcium citrate had previously been shown to result in a greater elevation in serum calcium than calcium carbonate [279] (although this was not the observed outcome in this trial (Chapter 3)). Blood coagulation was not measured on three participants due to technical problems with the device, so results are available for 35 participants. Blood coagulation was measured using a TEG 5000 Thromboelastograph Hemostasis Analyzer (Haemoscope Corporation, Niles, IL), at baseline, and 2, 4, 6 and 8 hours after the first dose of allocated treatment. At each time-point 2-3 ml of blood was drawn from a venous cannula into a syringe and discarded. A further 1-2 ml of blood was then drawn into a syringe and gently transferred to a test tube. From this, 360 µl of blood was transferred into a pre-warmed cup loaded into the TEG device. The TEG tracing was started 4 minutes after the blood was drawn from the cannula. R-time, K-time, alpha-angle, maximum amplitude and coagulation-index were recorded. The coagulation-index was calculated automatically by the device using the following equation:

$$\text{Coagulation index} = -0.1227\ \text{R-time} + 0.0092\ \text{K-time} + 0.1655\ \text{maximum-amplitude} - 0.0241\ \text{alpha-angle} - 5.022$$

Each of these measures represents an aspect of the coagulation system, although the exact processes underlying these measures are unclear. As described in the manufacturer's guidelines, R-time is a measure of the period of latency from the time the sample was placed in the device until initial fibrin formation; K-time is a measure of the time taken for the clot to reach a certain level of strength; alpha-angle is a measure of the rapidity of fibrin build-up and cross-linking; and maximum-amplitude is a measure the strength of the final clot [213]. Hypercoagulability is represented by a lower R-time or K-time and/or a greater alpha-angle or maximum-amplitude. Hypocoagulability is represented by changes in the opposite direction. The coagulation index describes a patients overall coagulation status, with more positive values indicating hypercoaguability and more negative values indicating hypocoaguability.

Regulators of vascular calcification

Regulators of vascular calcification were measured on participants allocated to calcium carbonate or placebo. The calcium carbonate group was selected for these measurements as it was possible citrate might interfere with the ability of calcium to precipitate. Regulators of vascular calcification were measured in blood samples collected at baseline and 4 and 8 hours after the first dose of allocated treatment was ingested, and in a fasting blood sample collected after 3 months of supplementation. All samples were spun following collection and the serum and plasma stored at -70ºC until analysis, as described below.

Nephelometry

Nephelometry was performed in all 40 participants allocated to calcium carbonate and placebo. This assay was first described by Pasch et al [225]. Briefly, nephelometry, which measures the amount of laser light scatter in turbid solutions, was used to measure the spontaneous conversion of small, primary calciprotein particles in serum into larger secondary calciprotein particles. A greater delay in the transition time of primary to secondary calciprotein particles reflects a greater stability of primary calciprotein particles, and therefore a greater calcification inhibitory potential of serum. Two hundred measurements, 3 minutes apart, were made on each sample using an automated laser-based

microplate nephelometer. The assay was performed at 37ºC and at a pH of 7.40, and consisted of 80 µl of serum with the addition of 20 µl of saline, 50 µl of calcium and 50 µl of phosphate. Excel and GraphPad Prism software were used to produce non-linear regression curves, allowing determination of the one-half maximal transition time (T_{50}) of primary to secondary calciprotein particles for each sample. Each sample was measured in triplicate, and the mean of the three values used in analyses. Outlying T_{50} values within each triplicate were removed prior to analysis by the researcher who performed the measurements and who had no contact with participants, and was blinded to treatment allocation. In this case the mean of the remaining two values was used in analyses.

Fetuin-A

Fetuin-A was measured on the first 10 participants allocated to calcium carbonate and 10 allocated to placebo who completed the study. Fetuin-A was measured using the Biovendor human ELISA kit (Biovendor, Modrice, Czech Republic).

Pyrophosphate

Pyrophosphate was measured on the first 10 participants allocated to calcium carbonate and 10 allocated to placebo who completed the study. Plasma pyrophosphate was assayed as previously described [239], with some modifications as follows. Plasma (20 µl) was added to 100 µl of reaction buffer containing 90 mM KCl, 5 mM $MgCl_2$, 70 mM TRIS-HCl (pH 7.60), 10 µM NADPH, 3.7 µM UDPG, 0.25 units/ml UDPG pyrophosphorylase (Type X from baker's yeast), 2.5 units/ml phosphoglucomutase (from rabbit muscle), 0.5 units/ml glucose-6-phosphate dehydrogenase (Type XV from baker's yeast), and 0.15 µCi/ml [^{14}C]UDPG. ATP, ADP, and AMP, each at 0.1 mM, were added to prevent hydrolysis of [^{14}C]UDPG by ecto-nucleotidases. All water was treated with hydroxyapatite to remove contaminating pyrophosphate. After 30 min at 37^{o} C, 200 µl of 3 % activated charcoal was added on ice with occasional stirring to bind residual UDPG. After centrifugation, the radioactivity in 200 µl of supernatant was counted.

Fibroblast growth factor-23

FGF23 was measured on the first 10 participants allocated to calcium carbonate and 10 allocated to placebo who completed the study. FGF-23 was measured using the Kainos intact ELISA kit (Kainos Laboratories, Tokyo, Japan).

Table 4.1 Baseline cardiovascular characteristics of participants

	Calcium citrate n = 18	Calcium carbonate n = 20	MCH A n = 20	MCH B n = 19	Placebo n = 20
Blood pressure					
Systolic (mmHg)	130 (21)	129 (15)	132 (15)	137 (24)	126 (20)
Diastolic (mmHg)	72 (8)	73 (9)	72 (7)	69 (8)	72 (8)
Thromboelastography					
R-time (min)	17.6 (5.3)				17.1 (3.9)
K-time (min	9.0 (2.7)				9.3 (3.2)
Alpha-angle (degrees)	24.6 (6.4)				24.5 (7.2)
Maximum-amplitude (mm)	50.0 (5.7)				47.4 (6.1)
Coagulation-index	-1.5 (1.4)				-1.8 (1.4)
Regulators of vascular calcification					
T_{50} (min)		383.1 (96.7)			352.6 (89.1)
Fetuin-A* (µg/ml)		245.5 (24.9)			245.7 (37.9)
Pyrophosphate* (µM)		1.7 (0.9)			2.2 (0.7)
FGF23* (pg/ml)		63.1 (29.3)			50.2 (7.6)

T_{50} = half the maximal transition time of primary calciprotein particles in serum into secondary calciprotein particles; FGF23 = fibroblast growth factor-23.

* Measured on the first 10 participants allocated to calcium carbonate, and the first 10 participants allocated to placebo, who completed the study
Data are mean (standard deviation)

4.3 RESULTS

The baseline clinical and biochemical characteristics of participants are presented in Table 3.1 (Chapter 3). The baseline cardiovascular characteristics of participants are presented in Table 4.1.

Blood pressure

Systolic blood pressure

Changes in systolic blood pressure over 8 hours and after 3 months are presented in Figure 4.1. Systolic blood pressure was lower than baseline at all time-points between 2 and 8 hours in the control group (all time-points $p < 0.0003$) and the MCH A group (all $p < 0.02$), between 2 and 6 hours in the calcium carbonate group (all $p < 0.03$), between 6 and 8 hours in the calcium citrate group (all $p < 0.005$) and at 2 and 6 hours in the MCH B group (all $p < 0.001$). The change in systolic blood pressure over 8 hours was significantly different at some time points between the groups (Figure 4.1). After 3 months of supplementation, systolic blood pressure was not different from baseline in any group. To examine the effect of calcium supplementation on blood pressure, the results from the four calcium treated groups were pooled and compared with the control group for subsequent analyses.

Changes in systolic blood pressure in the pooled calcium supplement group and the control group are presented in Figure 4.2. Systolic blood pressure was lower than baseline at all time-points from 2 to 8 hours in the control group (all time-points $p < 0.0003$) and the calcium supplement group (all $p < 0.0003$). The maximal reduction in systolic blood pressure in the control group was 17 mmHg, and was reached at 6 hours. Systolic blood pressure remained lower than baseline in the control group by 10 mmHg at 8 hours. The maximal reduction in systolic blood pressure in the calcium supplement group was 13 mmHg, and was reached at 6 hours. Systolic blood pressure remained lower than baseline in the calcium supplement group by 6 mmHg at 8 hours.

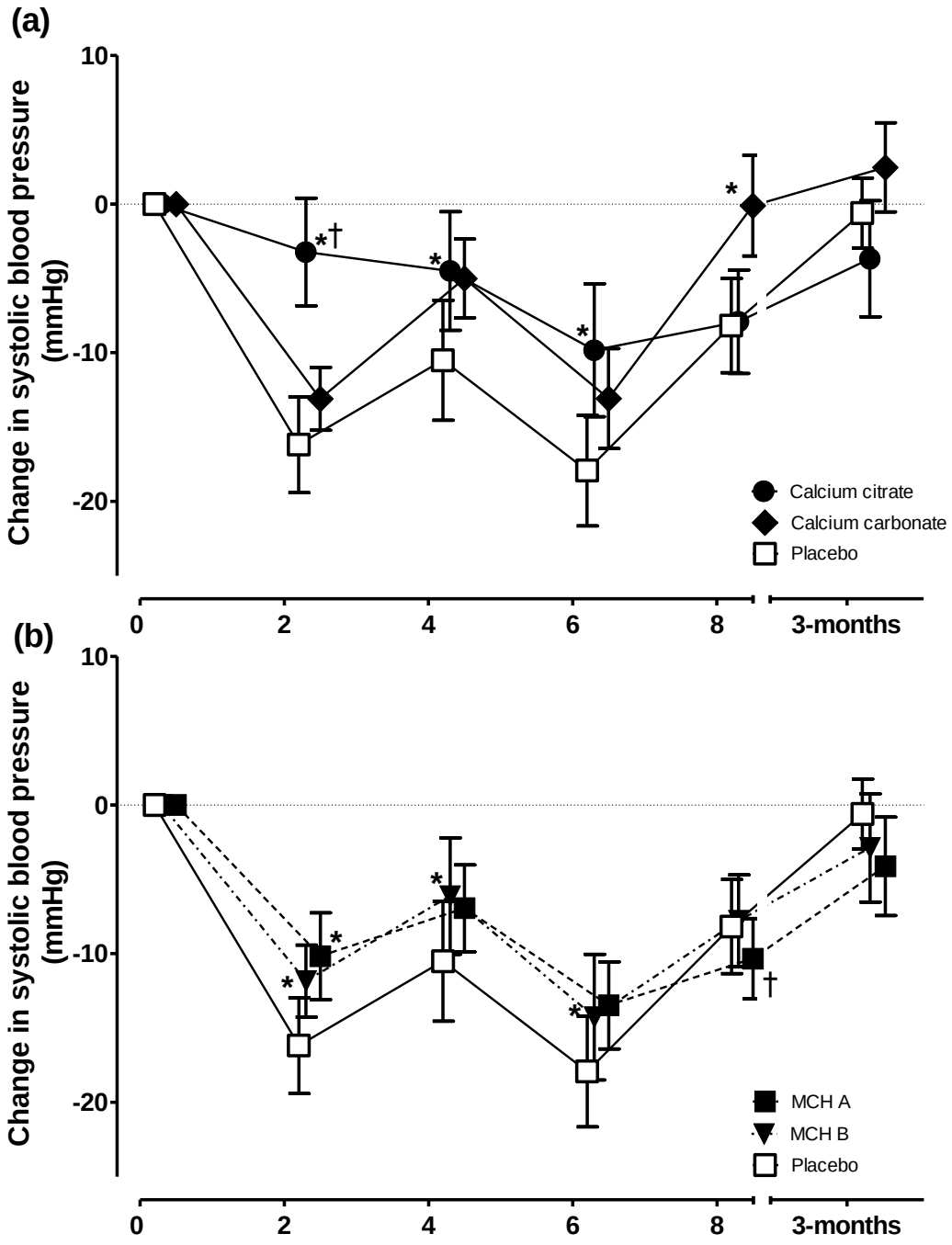

Figure 4.1 Change in systolic blood pressure over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 19) or MCH B (n = 19), or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and MCH B in graph b. Changes after placebo are shown in both graphs. The change in systolic blood pressure was significantly different among the five groups (ANCOVA, treat x time interaction, $p = 0.03$). *Significantly different from control in post-hoc tests, $p < 0.05$; † significantly different from calcium carbonate in post-hoc tests, $p < 0.05$. Values are mean ± SEM.

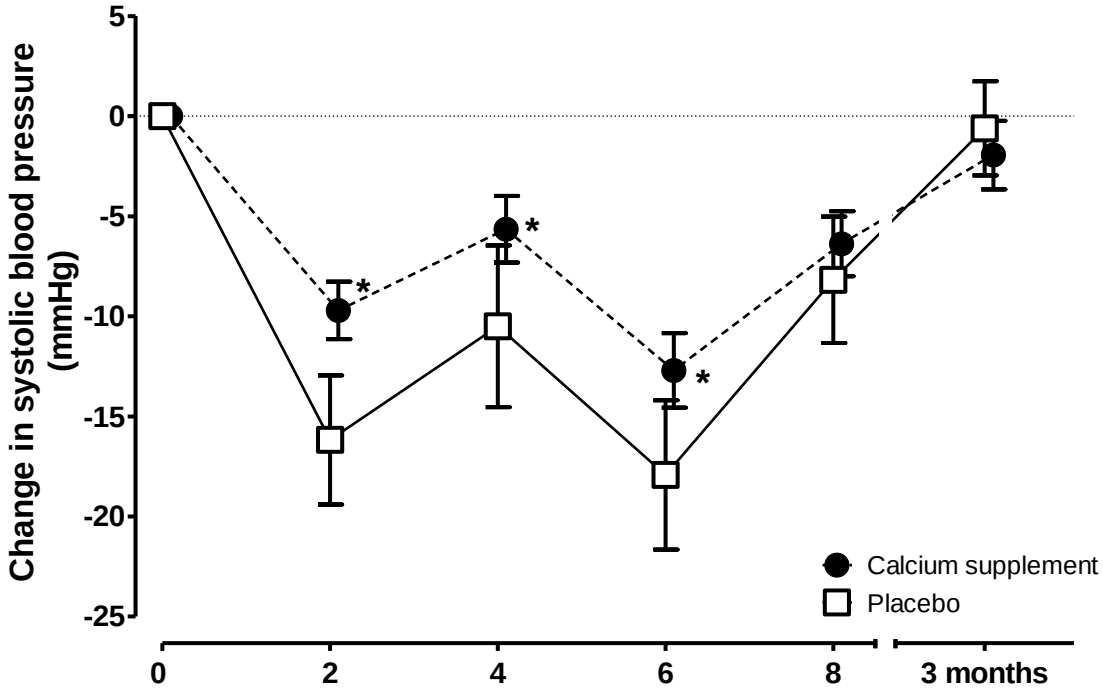

Figure 4.2 Change in systolic blood pressure over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate, carbonate or MCH (calcium supplement; n = 76) or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. The change in systolic blood pressure was not significantly different between the two groups over the whole study period (ANCOVA, treatment x time interaction, $p = 0.21$) but was significantly different from 2 to 6 hours on post-hoc testing, * $p < 0.05$. Values are mean ± SEM.

Diastolic blood pressure

Changes in diastolic blood pressure over 8 hours and after 3 months are presented in Figure 4.3. Diastolic blood pressure was lower than baseline at all time-points between 2 and 8 hours in the control group (all time-points $p < 0.0005$), between 2 and 6 hours in the calcium carbonate (all $p < 0.003$) and MCH A groups (all $p < 0.03$), at 2 and 6 hours the MCH B group (all $p < 0.0001$), and at 6 hours in the calcium citrate group ($p < 0.0001$). After 3 months of supplementation, diastolic blood pressure was lower than baseline in the MCH A group ($p = 0.046$), but not different from baseline in any other group. Changes in diastolic blood pressure were different at some time-points between the groups (Figure 4.3). As for systolic pressure, the results from the four calcium treated groups were pooled and compared with the control in subsequent analyses.

Changes in diastolic blood pressure in the pooled calcium supplement group and the control group are presented in Figure 4.4. Diastolic blood pressure was lower than baseline at all time-points from 2 to 8 hours in the control group (all time-points $p < 0.0006$) and the calcium supplement group (all $p < 0.0005$). The maximal reduction in diastolic blood pressure in the control group was 12 mmHg, and was reached at 6 hours. Diastolic blood pressure remained lower than baseline in the control group by 5 mmHg at 8 hours. The maximal reduction in diastolic blood pressure in the calcium group was 9 mmHg, and was reached at 6 hours. Diastolic blood pressure remained lower than baseline in the control group by 3 mmHg at 8 hours.

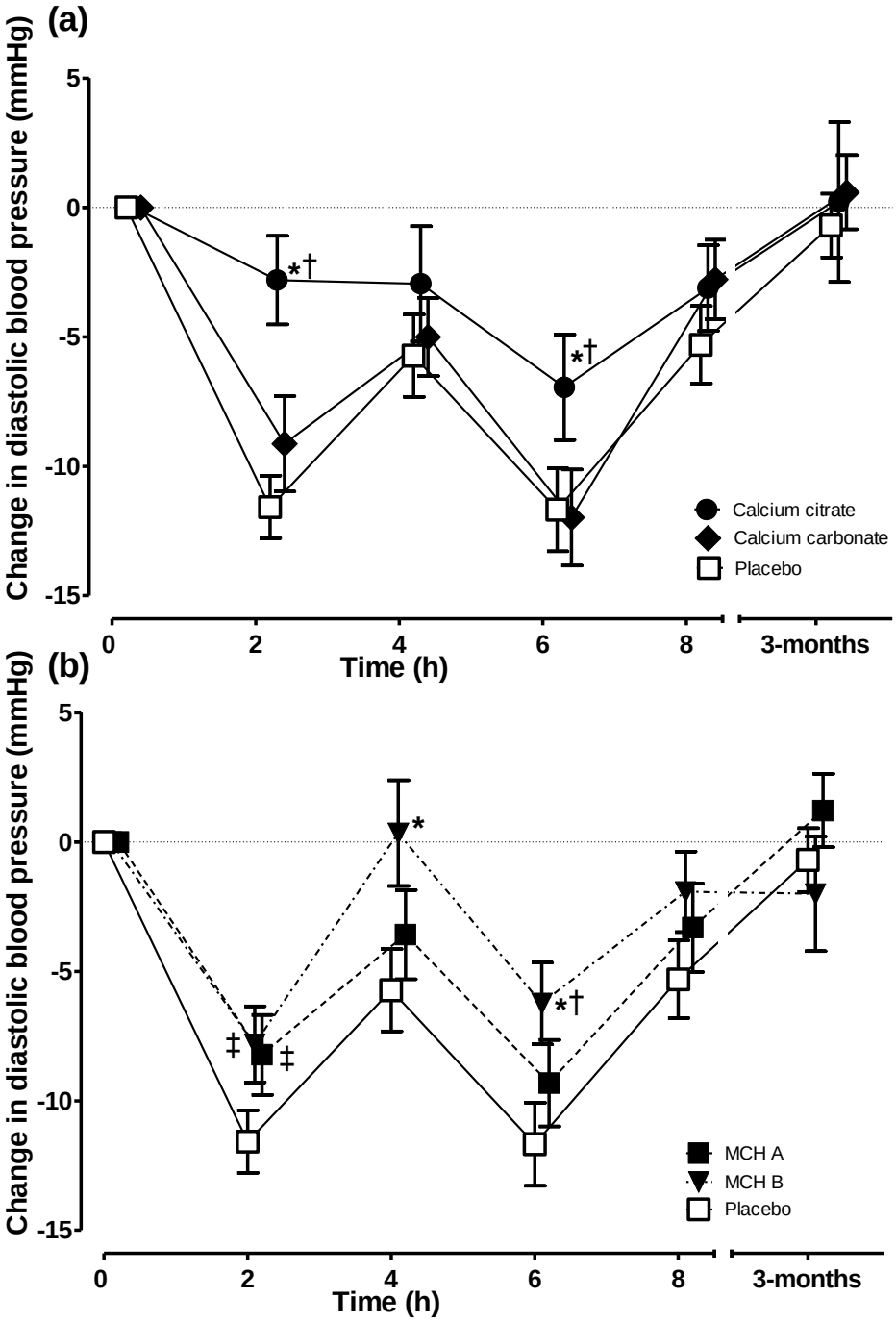

Figure 4.3 Change in diastolic blood pressure over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 18), carbonate (n = 20), MCH A (n = 19), MCH B (n = 19), or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. Changes after calcium citrate and calcium carbonate are shown in graph a, and after MCH A and MCH B in graph b. Changes after placebo are shown in both graphs. The change in diastolic blood pressure was significantly different among the five groups (ANCOVA, treat x time interaction, p = 0.005). *Significantly different from control in post-hoc tests, p <0.05; † significantly different from calcium carbonate in post-hoc tests, p <0.05; ‡ significantly different from calcium citrate in post-hoc tests, p <0.05. Values are mean ± SEM.

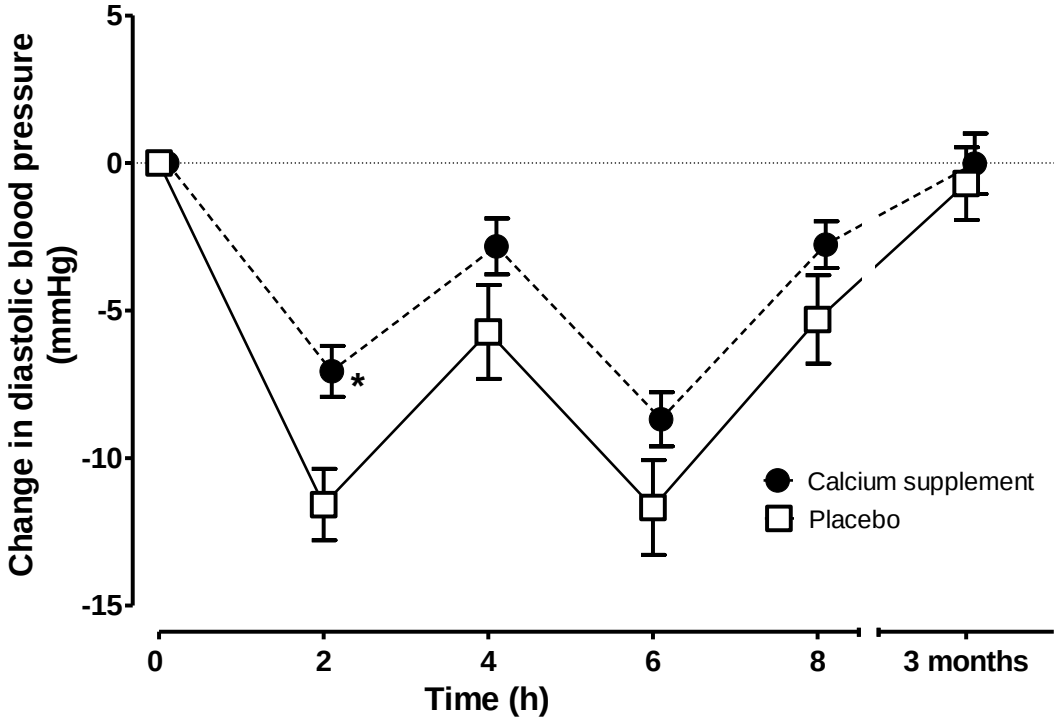

Figure 4.4 Change in diastolic blood pressure over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate, carbonate or MCH (n = 76) or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. The change in diastolic blood pressure was not significantly different between the two groups over the whole study period (ANCOVA, treat x time interaction, p = 0.32) but was significantly at 2 hours on post-hoc testing, * p = 0.01. Values are mean ± SEM.

Blood coagulation

R-time

Changes in R-time (the time to clot initiation) over 8 hours in the calcium citrate and control groups are presented in Figure 4.5. R-time was lower than baseline at all time-points between 2 and 8 hours in the calcium citrate group (all time-points $p < 0.003$), and between 4 and 8 hours in the control group (all $p < 0.04$). The maximal reduction in R-time in the control group was 3.6 minutes, and was reached at 8 hours. The maximal reduction in R-time in the calcium citrate group was 6.2 minutes, and was reached at 4 hours. R-time remained lower than baseline in the calcium citrate group by 4.7 minutes at 8 hours.

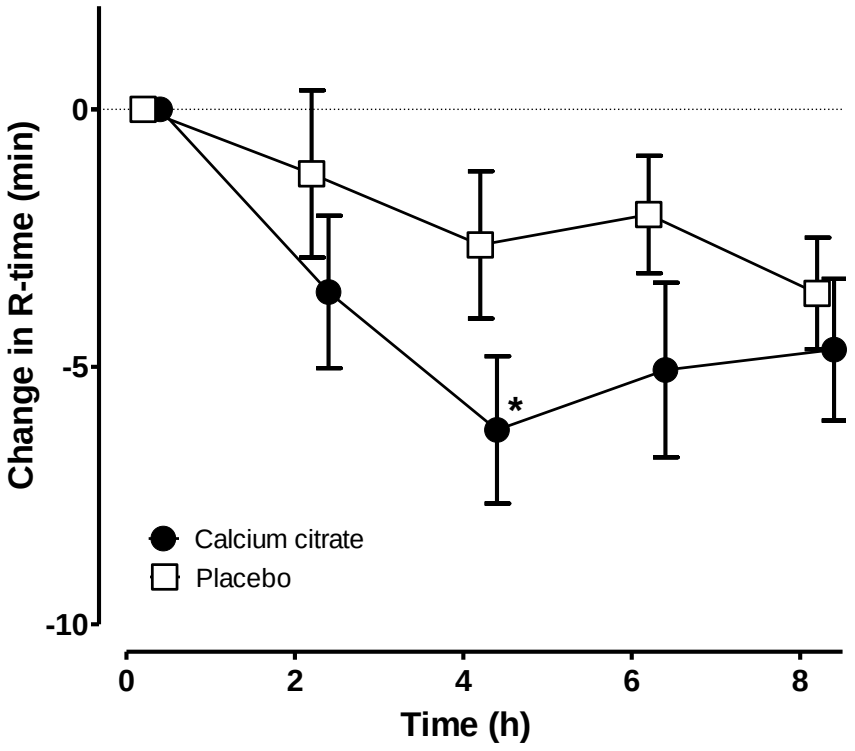

Figure 4.5 Change in R-time over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 16) or a placebo containing no calcium (control; n = 20). The change in R-time was not significantly different between the groups over the whole study period (ANCOVA, treatment x time interaction, $p = 0.31$) but was significantly different at 4 hours on post-hoc testing, * $p = 0.03$.

K-time

Changes in K-time (the time for the clot to reach a pre-defined level of strength) over 8 hours are presented in Figure 4.6. K-time was lower than baseline at all time-points between 2 and 8 hours in the calcium citrate group (all time-points $p < 0.01$), and at 4 and 8 hours in the control group (all $p < 0.01$). The maximal reduction in K-time in the control group was 1.6 minutes, and was reached at 4 and 8 hours. The maximal reduction in K-time in the calcium citrate group was 2.4 minutes, and was reached at 4 hours. K-time remained lower than baseline in the calcium citrate group by 1.9 minutes at 8 hours.

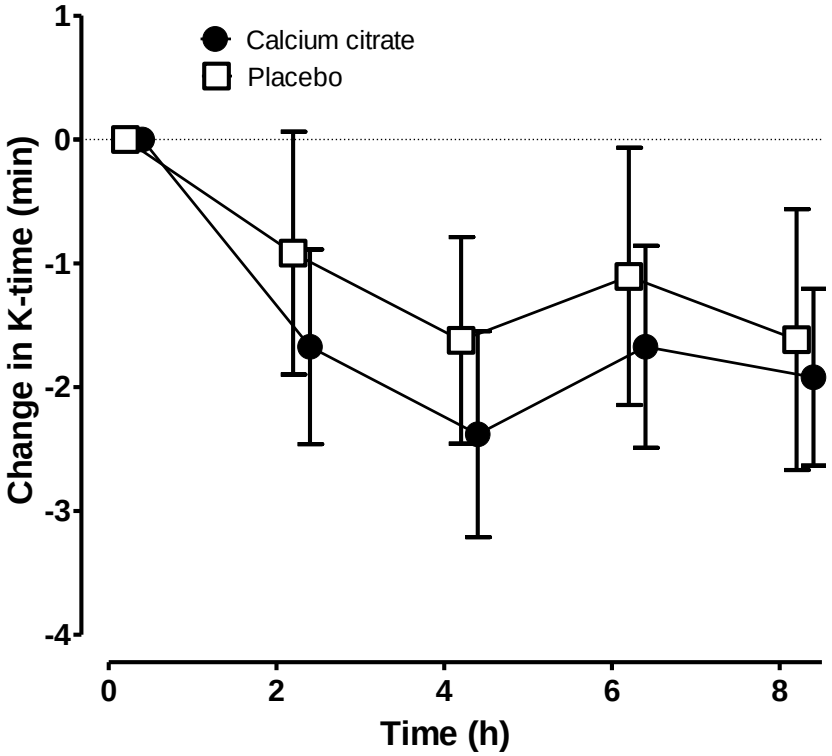

Figure 4.6 Change in K-time over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 16) or a placebo containing no calcium (control; n = 20). The change in K-time over 8 hours was not significantly different between the groups (ANCOVA, treatment x time interaction, p = 0.97).

Alpha-angle

Changes in alpha-angle (a measure of the rate of clot strengthening) over 8 hours are presented in Figure 4.7. Alpha-angle was increased from baseline at all time-points between 2 and 8 hours in the calcium citrate group (all $p < 0.01$), and at 4 and 8 hours in the control group (all $p < 0.02$). The maximal increase in alpha-angle in the control group was 6.6 degrees, and was reached at 4 hours. Alpha-angle remained higher than baseline in the control group by 5.3 degrees at 8 hours. The maximal increase in alpha-angle was 9.1 degrees in the calcium citrate group, and was reached at 4 hours. Alpha-angle remained higher than baseline in the calcium citrate group by 7.3 degrees at 8 hours.

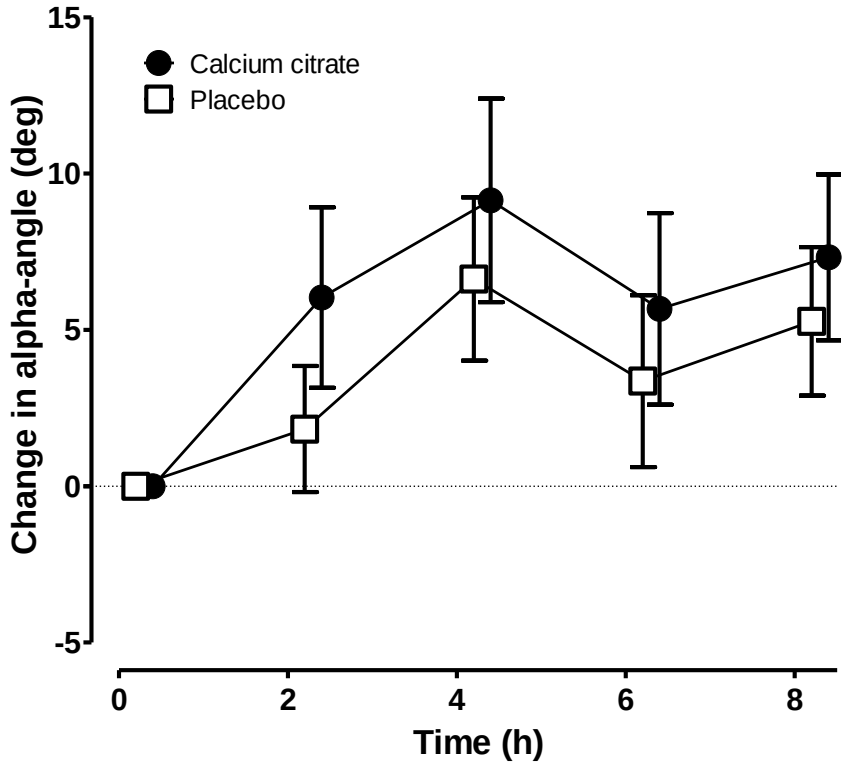

Figure 4.7 Change in alpha-angle over 8 hours in postmenopausal women after the ingestion of 1000 mg of calcium as citrate (n = 16) or a placebo containing no calcium (control; n = 20). The change in alpha-angle over 8 hours was not significantly different between the groups (ANCOVA, treatment x time interaction, $p = 0.86$).

Maximum-amplitude

Changes in maximum-amplitude (a measure of strength and size of the final clot) over 8 hours are presented in Figure 4.8. Maximum-amplitude was increased from baseline at 8 hours in the calcium citrate group ($p = 0.006$) and at no time-point in the control group. The maximal increase in maximum-amplitude in the calcium citrate group was 5.3 mm, and was reached at 8 hours.

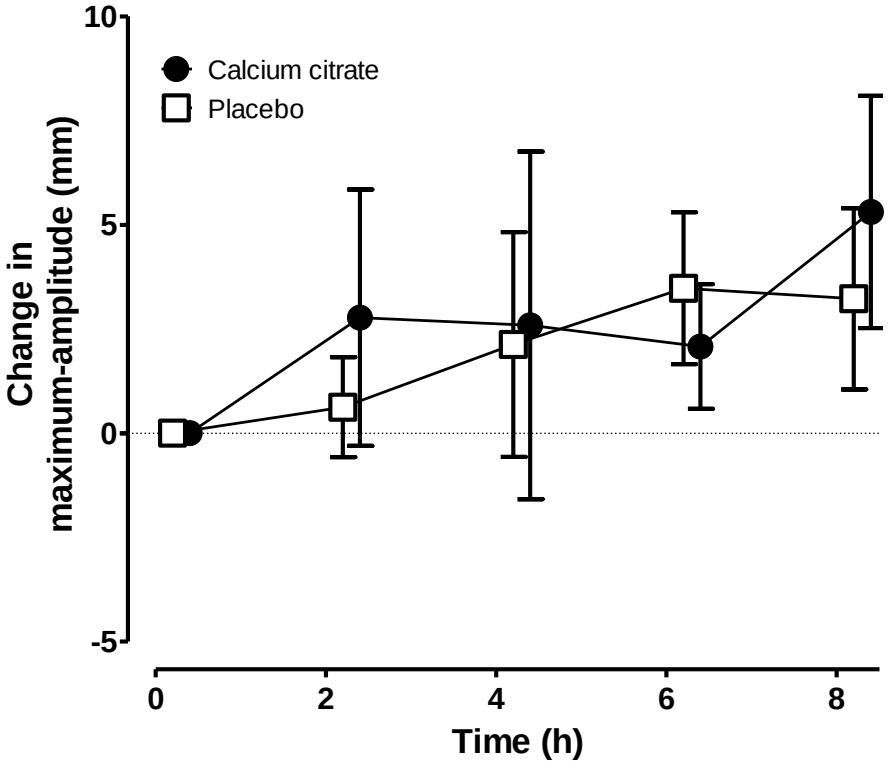

Figure 4.8 Change in maximum-amplitude over 8 hours in postmenopausal women after the ingestion of a 1000 mg of calcium as citrate (n = 16) or a placebo containing no calcium (control; n = 20). The change in maximum-amplitude over 8 hours was not significantly different between the groups (ANCOVA, treatment x time interaction, $p = 0.85$).

Coagulation-index

Changes in the coagulation-index (an overall assessment of coagulation) over 8 hours are presented in Figure 4.9. The coagulation-index increased from baseline at all time-points between 2 and 8 hours in the calcium citrate group (all $p < 0.006$), and between 4 and 8 hours in the control group (all $p < 0.04$). The maximal increase in the coagulation-index in the calcium citrate group was 2.3, and was reached at 4 hours. The maximal increase in the coagulation-index in the control group was 1.3, and was reached at 8 hours. The coagulation-index remained higher than baseline in the calcium citrate group by 1.8 at 8 hours.

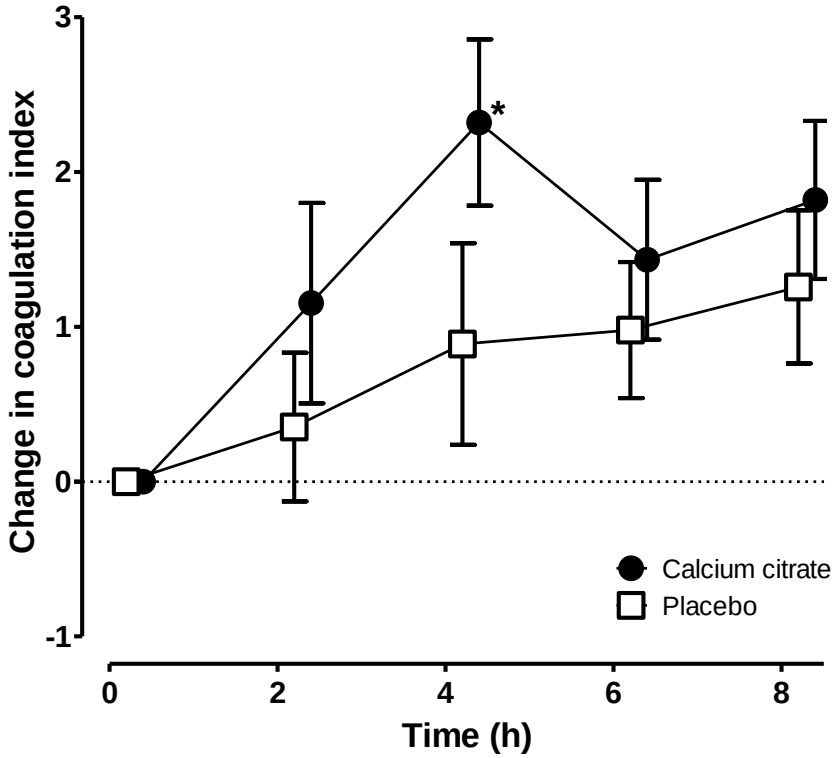

Figure 4.9 Change in coagulation-index over 8 hours in postmenopausal women after the ingestion of a 1000 mg of calcium as citrate (n = 16) or a placebo containing no calcium (control; n = 20). The overall change in coagulation index over 8 hours was not significantly different between the groups over the whole study period (ANCOVA, treatment x time interaction, p = 0.54) but was significantly different at 4 hours on post-hoc testing, * p = 0.03.

Nephelometry

Changes in T_{50} in the calcium carbonate and control groups are presented in Figure 4.10. T_{50} was lower than baseline at 4 hours, 8 hours and 3 months in the calcium carbonate (all time-points $p < 0.0005$) and control groups (all $p < 0.005$). The maximal reduction in T_{50} in the control group was 35 minutes and was reached at 4 hours. The maximal reduction in T_{50} in the calcium carbonate group was 67 minutes and was reached at 8 hours. After 3 months of supplementation T_{50} was lower than baseline by 20 minutes in the control group and by 52 minutes in the carbonate group.

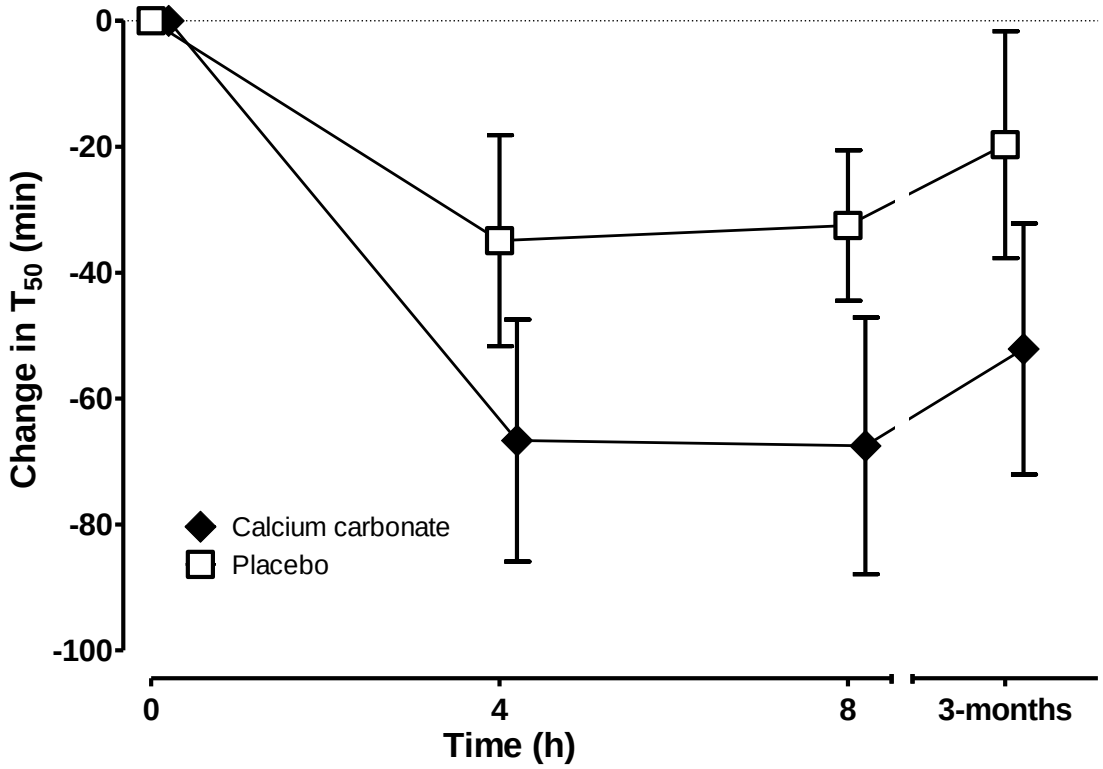

Figure 4.10 Change in T_{50} in postmenopausal women 4 and 8 hours after the ingestion of 1000 mg of calcium as carbonate (n = 20) or a placebo containing no calcium (control; n = 20), and after 3 months of supplementation. The change in T_{50} was not significantly different between the groups (ANCOVA, treatment x time interaction, $p = 0.98$).

Fetuin-A

Changes in fetuin-A are presented in Figure 4.11. Fetuin-A was not significantly different from baseline at 4 or 8 hours in the carbonate or control groups, or after 3 months of supplementation. There were no differences between the carbonate and control group in the change in fetuin-A at any time-point.

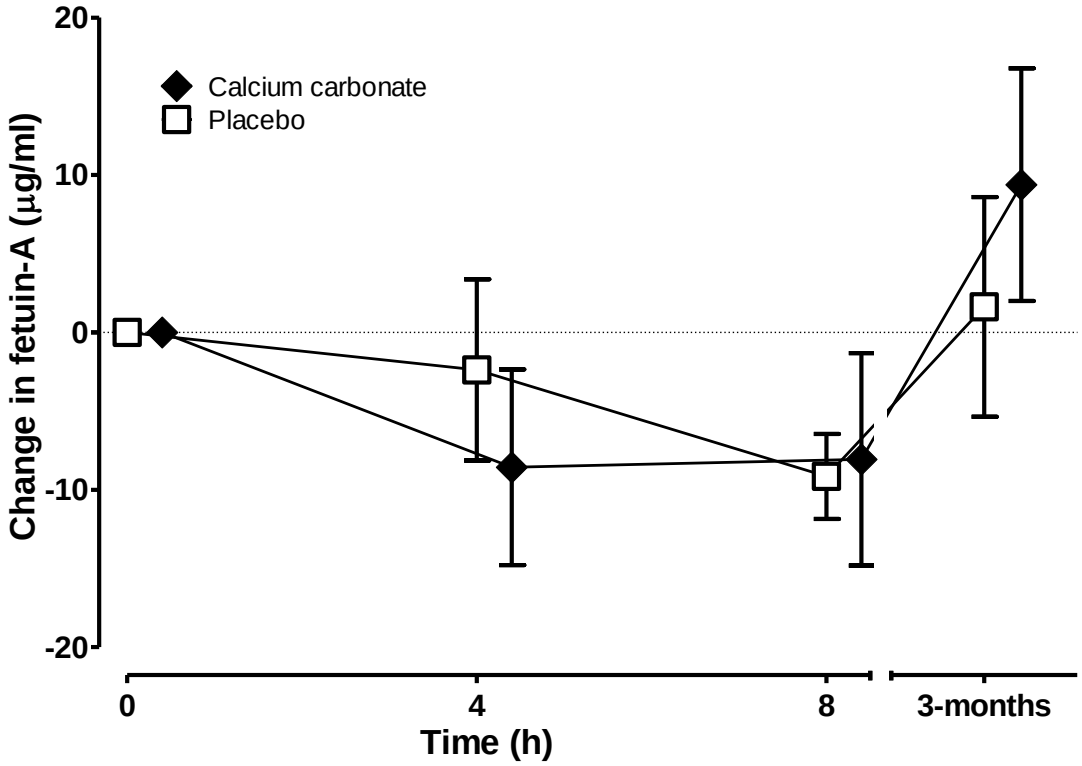

Figure 4.11 Change in fetuin-A in postmenopausal women 4 and 8 hours after the ingestion of 1000 mg of calcium as carbonate (n = 10) or a placebo containing no calcium (control; n = 10), and after 3 months of supplementation. The change in fetuin-A was not significantly different between the groups (ANCOVA, treatment x time interaction, p = 0.59).

Pyrophosphate

Changes in pyrophosphate are presented in Figure 4.12. Pyrophosphate was lower than baseline at 4 hours in the control group by 0.53 µM ($p = 0.002$), and was not different from baseline at 8 hours or after 3 months. Pyrophosphate was not different from baseline in the calcium carbonate group at any time-point.

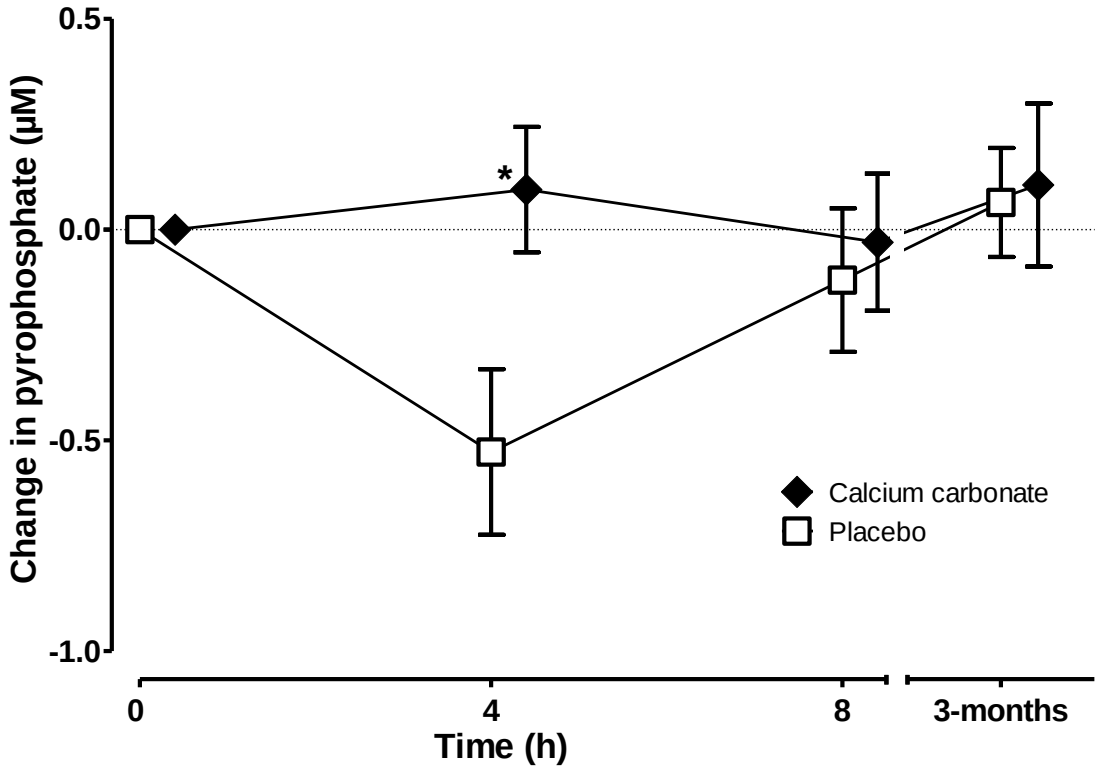

Figure 4.12 Change in pyrophosphate in postmenopausal women 4 and 8 hours after the ingestion of 1000 mg of calcium as carbonate (n = 10) or a placebo containing no calcium (control; n = 10), and after 3 months of supplementation. The overall change in pyrophosphate between the groups did not reach significance over the whole study period (ANCOVA, treatment x time interaction, $p = 0.07$), but was significantly different at 4 hours on post-hoc testing, $p = 0.04$.

Fibroblast growth factor-23

Changes in FGF23 are presented in Figure 4.13. FGF23 was not different from baseline at 4 or 8 hours in the carbonate or control groups. After 3 months of supplementation, FGF23 was increased from baseline by 4.35 pg/ml in the control group ($p = 0.03$) and by 4.53 pg/ml in the carbonate group ($p = 0.05$).

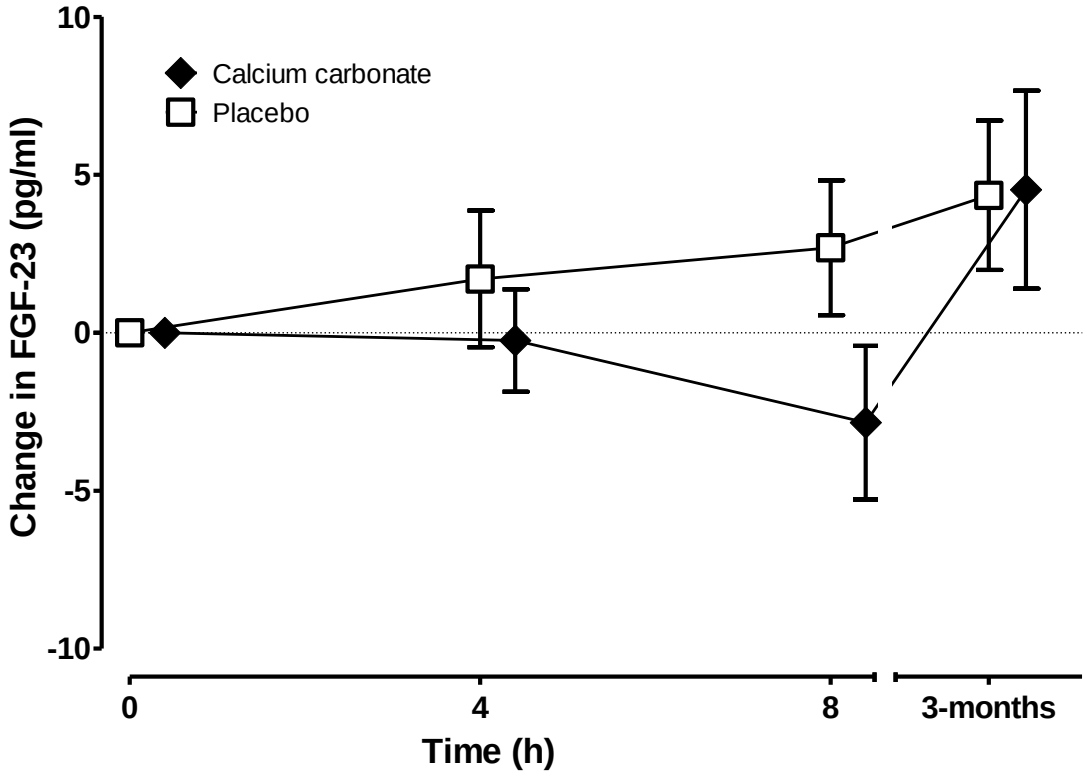

Figure 4.13 Change in FGF23 in postmenopausal women 4 and 8 hours after the ingestion of 1000 mg of calcium as carbonate (n = 10) or a placebo containing no calcium (control; n = 10), and after 3 months of supplementation. The change in FGF23 was not significantly different between the groups (ANCOVA, treatment x time interaction, $p = 0.59$).

4.4 DISCUSSION

In the present study there were adverse trends in blood pressure and blood coagulation acutely following the ingestion of a calcium supplement, which were significant at some time-points. Calcium supplementation had no effect regulators of vascular calcification acutely, or after 3 months of supplementation, although the number of participants in those analyses was small. The adverse trends in blood pressure and blood coagulation might contribute to the increased cardiovascular risk associated with calcium supplementation [29]. However, due to the small number of participants and borderline significance of these findings, they require investigation in larger trials.

Blood pressure

Blood pressure declined from baseline over 8 hours in both the calcium supplement and control groups. This might reflect a diurnal rhythm in blood pressure, which has been reported to peak mid-morning and decline thereafter [303]. A meal has also been shown to lower blood pressure by around 15 mmHg [304], similar to the change in blood pressure from baseline in the present study and possibly reflecting splanchnic vasodilation [305]. Alternatively, the higher baseline measurement may have reflected white-coat hypertension, which resolved in the subsequent measurements [306].

The reduction in blood pressure over 8 hours tended to be smaller in the calcium supplement group compared with the control group, and the difference was significant at some time-points. This difference in blood pressure might be explained by the elevation in serum calcium that occurred in the calcium supplemented groups (Chapter 3). Nilsson et al demonstrated that a calcium infusion which increased ionised calcium by 0.32 mmol/l reduced endothelial vasodilatory function, and increased systolic blood pressure from 114 to 121 mmHg [194]. Similarly, Kamycheva reported that a calcium infusion which increased ionised calcium by 0.20 mmol/l increased systolic blood pressure from 123 to 134 mmHg [195]. Furthermore, in patients undergoing haemodialysis, the use of a higher calcium dialysate resulted in less of a reduction in intradialytic blood pressure [200-204]. In one study, the use of a low versus high calcium dialysate resulted in post-dialysis ionised calcium concentrations of 1.11 and 1.19 mmol/l, systolic blood pressures of 134 and 145 mmHg and

diastolic blood pressures of 68 and 74 mmHg, respectively [200]. These effects have been attributed to changes in myocardial contractility, stroke volume and arterial stiffness [200, 202-206].

The trend for higher blood pressure in the calcium supplement group relative to the control group in the present study is in contrast with previous longer-term trials of calcium supplements, which have demonstrated modest reductions in blood pressure [153-156, 159]. Differences between the present study and previous studies might be explained by the timing of the blood pressure measurement relative to the dosing with calcium. In two calcium supplement trials by Reid et al [153, 159], blood pressure was measured fasting the morning after an evening dosing. Other studies may have used a similar protocol. As serum calcium returns to baseline 6 to 12 hours after the ingestion of a supplement [69, 70, 178], these studies could have missed any potential change in blood pressure associated with the acute rise in serum calcium that follows the ingestion of a supplement.

To my knowledge, only two studies have examined blood pressure acutely following the ingestion of a calcium supplement. In a study of 25 older men and women, serum calcium increased from baseline 3 hours after the ingestion of 1000 mg of supplemental calcium, but blood pressure did not change [207]. In small study of 11 men and women, serum calcium increased 2 hours after the ingestion of 600 mg of supplemental calcium, but again, blood pressure did not change [208]. However, the small size, lack of a control group, and measurement of blood pressure only at one time-point limits the interpretation of their findings. In the present study, blood pressure was only elevated in the calcium supplement group relative to the control group, not to baseline measurements.

After 3 months of supplementation, blood pressure was not different from baseline in the calcium supplement or control groups. This is broadly consistent with other long-term trials, which have reported only small reductions in blood pressure with calcium supplementation, typically in the range of 0.5 – 2.5 mmHg [154-156]. The size of the present study means it is likely it would have been underpowered to detect a small effect on blood pressure, if it were present.

Blood coagulation

Blood coagulability increased over 8 hours in the calcium citrate and control groups. This was reflected by a decrease in R-time and K-time, and an increase in alpha-angle and maximum-amplitude. The coagulation-index, which is based on all four parameters and describes a patient's overall coagulation, correspondingly increased from baseline in both groups. There was a greater decrease in R-time (the time to clot initiation) at 4 hours, and greater increase in the coagulation-index at 4 hours, in the calcium citrate group relative to the control group. Of note, the maximal increase in ionised and total calcium was also reached at the 4 hour time-point (Chapter 3).

Little research has investigated whether serum calcium, above the threshold required for coagulation to occur, influences blood coagulation. Three previous studies have used TEG to assess the effects of changes in serum calcium on coagulation. In rats, experimentally induced hypercalcaemia significantly shortened the R-time [218]. In human blood samples citrated and re-calcified with varying amounts of calcium, ionised calcium above 0.56 mmol/l was inversely associated with R-time and positively associated with maximum-amplitude [219]; although the relationship appeared to be strongest at an ionised calcium concentration below the normal range in blood. In a similar study using citrated and re-calcified human blood samples, blood coagulability increased up to an ionised calcium concentration of 2.1 mmol/l [307]. These results should be interpreted with caution, however, as citrated and re-calcified blood samples may not reliably reflect coagulation in native whole blood [220, 221]. The present study is the first to examine the effect of small changes in serum calcium on blood coagulation in native whole blood.

Which of several possible mechanisms could mediate a potential effect of serum calcium on blood coagulation is presently unclear. Platelets express the calcium-sensing receptor [210], and increased serum calcium might therefore influence platelet activation or aggregation. Calcium is required for several steps in the coagulation cascade, including the conversion of prothrombin to thrombin, however this is currently understood be a threshold effect, which is exceeded at concentrations well below the normal range in blood [211]. A pro-coagulant effect of calcium may also be mediated through an increased ratio of serum calcium to magnesium, rather than increased serum calcium per se. Magnesium infusions were shown to

attenuate the hypercoagulability associated with surgery [308] and preecamplsia [309], which was suggested to be due to an antagonistic effect of magnesium on calcium.

A potential limitation of the present study is the method used to assess clotting. TEG is subject to several pre-analytical and analytical issues that may influence results (e.g. differences in the time-point after sample collection at which TEG analysis is performed), and as a result, its *inter*-laboratory reproducibility has yet to be proven [310]. However, as the measurements in the present study were from a single operator following a strict protocol based on the manufacturer's guidelines, variability should be minimised. Further research is needed to establish the reproducibility and consistency of TEG measures of blood coagulation.

Regulators of vascular calcification

There were no differences between the calcium carbonate and control groups in the propensity of serum to calcify, or in three suspected regulators of vascular calcification: fetuin-A, pyrophosphate and FGF23. The one exception was a reduction in pyrophosphate at 4 hours in the control group, which did not occur in the calcium carbonate group.

Nephelometry

The method used in our trial to assess the calcification propensity of serum was first described by Pasch et al [225]. This test is based on the time taken for small, primary calciprotein particles in serum to transition into larger, crystalline, secondary calciprotein particles (denoted T_{50}). A greater T_{50} reflects a greater ability of serum to withstand calcification. Pasch et al reported that fetuin-A, albumin and magnesium had calcification-inhibitory effects (increased T_{50}), and phosphate and calcium calcification-promoting effects (decreased T_{50}), although the effect of calcium was relatively weak [225]. Recently, Smith et al reported that T_{50} was inversely associated with ionised calcium in patients with CKD [227]. They also reported that calcium supplement use was associated with a lower T_{50}, although the number of individuals who were on therapy was small (n = 23). In contrast, in the present study, T_{50} was not different between the carbonate and control groups acutely, or

after 3 months of supplementation. There did appear to be a greater reduction in T_{50} at all time-points in the calcium carbonate group compared with the control group, however this was not significant, possibly due to the small number of participants.

There was a reduction in T_{50} from baseline over 8 hours in both the control and calcium carbonate groups in the present study. This might be related to diurnal changes in promoters and inhibitors of calcification, or differences in T_{50} between the fed and fasting state. T_{50} was also lower than baseline at 3 months in both groups. This might be related the timing of the 3 month blood sample, which was collected later in the morning than the baseline sample, although still fasting.

Fetuin-A

There were no significant differences in fetuin-A between the calcium carbonate and control groups in the present study. In CKD patients, decreased fetuin-A is associated with increased vascular calcification and mortality [230, 231]. In contrast, in the normal or pre-dialysis population, increased fetuin-A is associated with calcification and cardiovascular risk [232, 233]. This may indicate fetuin-A synthesis is initially up-regulated as a defense against the early stages of calcification [232]. Consistently, the administration of a vitamin D analog (paricalcitol) to hyperparathryoid patients on dialysis increased fetuin-A after 8 weeks of treatment [234] and in children with CKD, increased serum calcium and intakes of 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D were positively associated with fetuin-A [235]. Interestingly, in the present study, fetuin-A appeared to be higher in the carbonate group than the control group at 3-months; however, this was not significant, possibly due to the small number of participants.

Pyrophosphate

Lower plasma pyrophosphate concentrations are associated with increased calcification in patients with CKD [237]. In the present study, there was a significant reduction in pyrophosphate at 4 hours in the control group, which did not occur in the calcium carbonate group. This might have been a spurious finding, as the measurement of pyrophosphate is a

difficult assay [237]. Alternatively, this might reflect a diurnal rhythm in pyrophosphate, which was somehow suppressed in the carbonate group, although there is no evidence to support this. As only total pyrophosphate was measured in the present study, I cannot rule out that the increased serum calcium in the carbonate group may have complexed with and reduced the amount of free pyrophosphate.

Fibroblast growth factor-23

Calcium supplementation had no effect on FGF23 acutely, or after 3 months of supplementation. The regulation of FGF23 is incompletely understood, but 1,25-dihydroxyvitamin D and phosphate are both known to increase its concentration [248, 249]. In animal studies, calcium has also been shown to regulate FGF23, with dietary calcium supplementation increasing FGF23 [250] and diet-induced hypocalcaemia reducing its concentration [251]. The duration of treatment also appears to be important, since chronic phosphate loading increases FGF23 [311] but acute phosphate loading has no effect [312]. The findings of the present study suggest that FGF23 similarly does not respond acutely to calcium administration. There was a non-significant trend for FGF23 to decline over 8 hours in the calcium treated group, which if true, might have been related to the suppression 1,25-dihydroxyvitamin D, although this was not measured. After 3 months, FGF23 was increased from baseline to a similar degree in both the calcium and control groups. The reason behind this increase in unclear, but it may be a chance finding due to the small number of participants, or could be related to possible changes in vitamin D status during the study.

In summary, there was a trend for blood pressure to be higher following the ingestion of a calcium supplement, and blood coagulation increased, but these differences were only significant at certain time-points. Calcium supplementation had no significant effect on any of the regulators of vascular calcification measured, however, the numbers of participants were small, and this study may have been underpowered to detect a small effect of calcium on these outcomes. Further research is needed to examine the acute effects of calcium supplementation on blood pressure and blood coagulation. The results of the present trial could be used to inform the design of these studies.

CHAPTER 5: ACUTE EFFECTS OF CALCIUM CITRATE, CALCIUM-FORTIFIED JUICE AND A DAIRY MEAL ON SERUM CALCIUM: A RANDOMISED CROSS-OVER TRIAL

5.1 INTRODUCTION

In Chapter 3, I demonstrated that 1000 mg of supplemental calcium elevated serum calcium for at least 8 hours. However, in most trials included in the meta-analysis of calcium supplements and cardiovascular risk, calcium supplements were administered in two divided doses of 500 - 600 mg each day [30]. Any adverse effect of calcium supplements must therefore be related to the effects of a smaller, but more regular, dose of calcium than that in my previous trial. The effects of 500 mg of supplemental calcium are also clinically relevant, as calcium supplements are recommended to be taken in doses no larger than 500 mg, to maximise absorption [282].

In contrast to calcium supplementation, most observational evidence suggests that dietary calcium is not associated with increased cardiovascular risk [36, 170, 177]. A possible difference between dietary calcium and supplemental calcium is their effects on serum calcium. Supplemental calcium is consumed in a bolus of 500 – 1000 mg, once or twice a day, while dietary calcium will be consumed in small amounts spread over a day (although single doses of 500 mg of calcium may be achieved through the use of calcium-fortified foods). Moreover, even large doses of dietary calcium appear to have a smaller calcaemic effect than equivalent doses of supplemental calcium [140, 141], although this has not been well-studied. This might be related to the protein and fat in dietary calcium sources slowing the rate at which calcium is released into the blood. For this reason, it has been suggested that calcium supplements should be taken with meals, in order to reduce their calcaemic effects [313, 314]. However, there is no evidence to support this advice, as in acute trials calcium supplements have only been administered fasting or with a light meal [68, 69, 71, 279]. Calcium-fortified foods may also be used to supplement the diet. Their effects on serum calcium appear to be similar to that of supplements [141, 315], but again, this has not been well studied.

As it is possible that the adverse cardiovascular effects of calcium supplements are related to their effects on serum calcium, the calcaemic effects of different calcium sources are of interest. I carried out a cross-over trial which examined the acute effects of 500 mg of calcium from a supplement on serum calcium, and whether this was different when a calcium supplement was taken with a meal high in protein and fat, or after calcium from fortified juice or a meal of dairy products.

5.2 METHODS

Participants

Participants were 10 women at least 5 years post menopause. I recruited women from those who had participated in the RCT described in Chapter 3. Thirty women were contacted by mail and invited to participate in the present study, of which ten agreed to do so. The inclusion and exclusion criteria were the same as for my previous study (Chapter 3). Briefly, women did not have osteoporosis or any other systemic disease, and were not taking any medication known to affect calcium or bone metabolism.

Protocol

Participants attended four 6-hour sessions at a research clinic, with each session separated by at least 7 days. At each session participants attended the clinic the morning after an overnight fast. A baseline blood sample was collected, and participants received one of the following four treatments in random order: 500 mg of elemental calcium as citrate, fasting; 500 mg of elemental calcium as a calcium-fortified fruit juice, fasting; 500 mg of elemental calcium as citrate, taken immediately after a meal (a ham and egg omelette with margarine and toast); or 500 mg of elemental calcium from a meal of unfortified dairy products (milk, yoghurt and cheese with toast). The four treatments and their composition are described in Table 5.1. A combination of dairy products, rather than a single food, was used to attempt to best represent a normal diet. The calcium-fortified juice was a commercially available product fortified with calcium as lactate and gluconate (Schweppes Australia Pty Ltd, Melbourne, Australia).

Calcium citrate (Jost Chemical Co., USA) was purchased from Hawkins Watts (Auckland, New Zealand).

Blood samples were collected 1, 2, 4 and 6 hours after each treatment was ingested. A light breakfast meal was provided after the calcium citrate fasting and fortified juice interventions, 1 hour after the treatment was ingested (Table 5.1). A light lunch meal (toast with jam, honey or marmalade and tinned fruit in juice) was provided after all interventions, 4 hours after the treatment was ingested. The lunch meal contributed a further 73 mg of calcium and 142 mg of phosphorous, by calculation. All meals were served after the sampling procedures were complete for that time-point. Water was allowed *ad libitum*, but no other food or drink was permitted. This study received ethical approval from the New Zealand Northern Regional X Ethics Committee (NTX/10/12/125). Written informed consent was obtained from all participants. This study was registered with the Australia New Zealand Clinical Trials Registry (ACTRN12614000342617).

Measurements

Ionised calcium was measured on anaerobically handled specimens using an ABL800 FLEX blood gas analyser (Radiometer, Bronshoj, Denmark). Samples for the measurement of total calcium and phosphate were batch-analysed at the end of each 6-hour session using a Coba modular analyser (Roche Diagnostics, Indianapolis, Indiana). PTH and markers of bone turnover were not assessed in the present study, as feeding impacts PTH and bone turnover [316, 317], and the interventions were balanced only in calcium content.

Body weight was measured using electronic scales, and height using a Harpenden stadiometer. Dietary calcium intake was assessed in my previous trial (Chapter 3) using a validated food frequency questionnaire [277].

Statistical analyses

Data were analysed on an intention-to-treat basis using a mixed models approach to repeated measures (Proc Mixed, SAS v 9.2, SAS Institute Inc). The change from baseline was the dependent variable and baseline value of the appropriate variable was included as a covariate

(ANCOVA). Significant main (time or treatment allocation) and interaction effects (time by treatment allocation) were further explored using the method of Tukey to construct honestly significant differences; however since these comparisons were pre-planned the pairwise P values were not adjusted for multiplicity. The areas under the ionised calcium, total calcium or phosphate over time functions (AUC) were calculated using a trapezoidal method and provided the dependent variable as indicated.

Table 5.1 Composition of interventions

Intervention and meal*	Serving size	Calcium (mg)	Phosphorous (mg)	Energy (kcal)	Fat (g)	Protein (g)	Carbohydrate (g)
Citrate-fasting							
Calcium citrate	500 mg	500	-	-	-	-	
One hour after the ingestion of calcium citrate:							
Wheat bread	84 g	64	118	160	2.2	7.6	27
Peaches in juice	115 g	7	20	46	0.1	0.7	10
	Total	**571**	**138**	**206**	**2.3**	**8.3**	**37**
Fortified-juice							
Calcium-fortified fruit juice	500 ml	500	40	220	0.1	1.5	53
One hour after the ingestion of fortified juice:							
Wheat bread	84 g	64	118	160	2.2	7.6	27
Peaches in juice	115 g	7	20	46	0.1	0.7	10
	Total	**571**	**178**	**426**	**2.4**	**9.8**	**90**
Citrate-with-a-meal							
Calcium citrate	500mg	500	-	-	-	-	
Eggs	2 eggs	58	212	152	10.6	13.6	0.4
Ham	50 g	0	34	52	2.6	8.4	1.0
Wheat bread	42 g	32	59	80	1.1	3.8	13
Margarine	20 g	2	2	134	14	0	0
	Total	**592**	**307**	**418**	**28.3**	**25.8**	**14.4**
Dairy-meal							
Milk (1.5% fat)	100 ml	128	96	47	1.5	3.5	9.6
Yoghurt (sweetened)	125 g	158	138	113	3.6	5.0	15.0
Cheese	34 g	230	193	143	12.2	8.4	0.1
Wheat bread	84 g	64	118	160	2.2	7.6	27
	Total	**580**	**545**	**463**	**19.5**	**24.5**	**51.7**

***** All meals were served with an option of jam, marmalade or honey, and decaffeinated tea or coffee without milk, which would have contributed an additional 2 mg of calcium and 4 mg of phosphorous**.**

5.3 RESULTS

The baseline characteristics of participants are presented in Table 5.2.

Table 5.2 Baseline clinical and biochemical characteristics of participants.

	N = 10
Clinical characteristics	
Age (y)	69 (3)
Weight (kg)	74 (13)
Height (m)	1.63 (0.06)
Dietary calcium (mg/day)	840 (400)
Biochemical characteristics	
25-hydroxyvitamin D (nmol/l)	70 (24)
Ionised calcium (mmol/l)	1.24 (0.03)
Total calcium (mmol/l)*	2.25 (0.05)
Phosphate (mmol/l)	1.08 (0.11)

Values are mean (standard deviation). *Total calcium corrected for albumin 40 g/l.

Ionised calcium

There were significant differences in the change in ionised calcium between the four interventions (ANCOVA, treatment x time interaction $p = 0.04$). Changes in ionised calcium were not significantly different between the citrate-fasting and fortified-juice interventions at any time-point (Figure 5.1). To simplify the comparison between the interventions, the results from citrate-fasting and fortified-juice interventions were pooled in subsequent analyses (as citrate-juice; Figure 5.2).

Ionised calcium was increased from baseline between 2 and 6 hours after citrate-juice (all time-points $p < 0.0001$), between 4 and 6 hours after citrate-with-a-meal (all $p < 0.003$), and at 2 hours after the dairy-meal ($p = 0.009$). The maximal increase in ionised calcium after citrate-juice was 0.03 mmol/l, and was reached at 4 and 6 hours; after citrate-with-a-meal was 0.03 mmol/l, and was reached at 6 hours; and after the dairy-meal was 0.02 mmol/l and was reached at 2 hours. There were significant differences between the interventions in the change in ionised calcium (Figure 5.2). One participant had an ionised calcium measurement above the normal reference range (greater than 1.30 mmol/l) 6 hours after citrate-juice, and one participant had an ionised calcium above than the normal reference range 4 hours after citrate-juice, 2 and 4 hours after citrate-with-a-meal, and 1 hour after the dairy-meal. The AUCs of ionised calcium are presented in Figure 5.3.

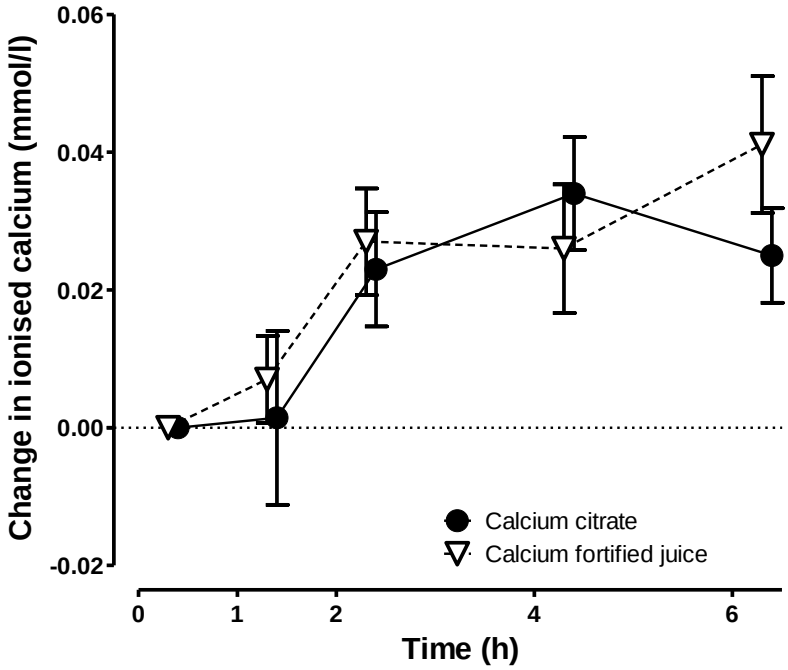

Figure 5.1 Change in serum ionised calcium over 6 hours in postmenopausal women (n = 10) after the ingestion of 500 mg of calcium citrate or 500 mg of calcium from fortified juice. Values are mean ± SEM.

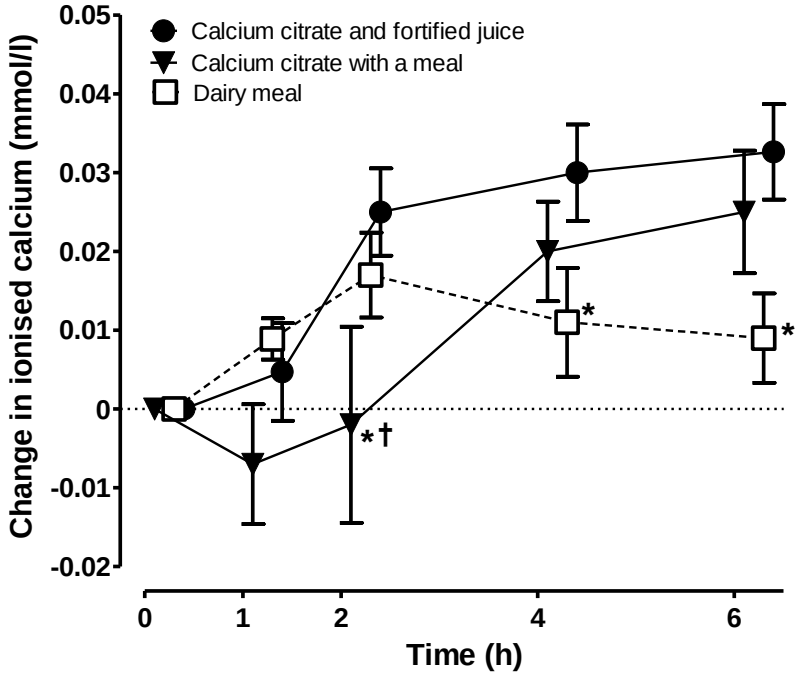

Figure 5.2 Change in serum ionised calcium over 6 hours in postmenopausal women (n = 10) after the ingestion of 500 mg of calcium citrate or fortified juice, 500 mg of calcium citrate with a meal and 500 mg of calcium from a dairy meal. Values are mean ± SEM. There was a significant treatment x time interaction, (ANCOVA, p = 0.048). * Significantly different from calcium citrate and fortified juice; † significantly different from the dairy meal, p <0.05.

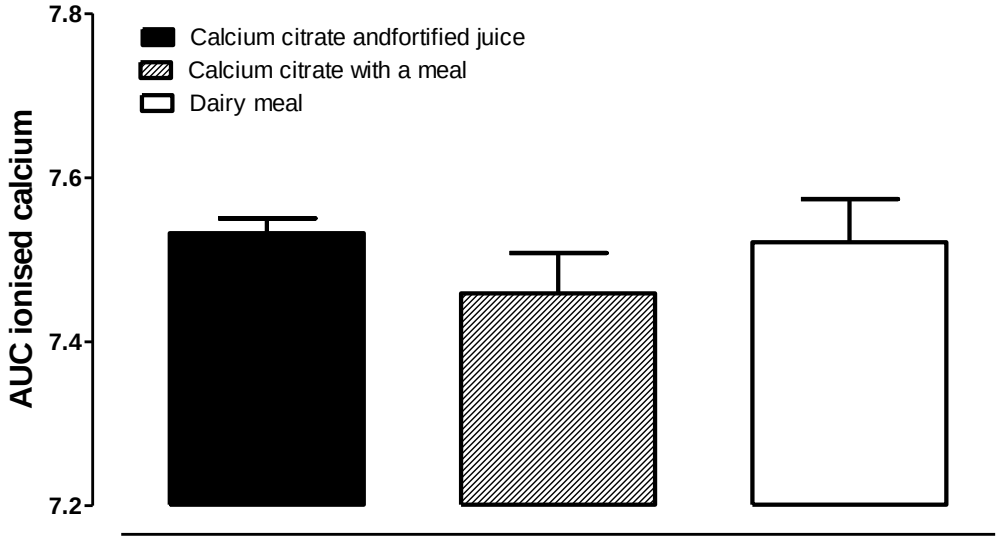

Figure 5.3 Area under the curve (AUC) of the change in serum ionised calcium in postmenopausal women (n = 10) over 6 hours after 500 mg of calcium citrate or fortified juice, 500 mg of calcium citrate with a meal or 500 mg of calcium from a dairy meal. The AUCs for ionised calcium were not significantly different between the interventions (p = 0.32).

Total calcium

There were significant differences in the change in total calcium between the four interventions (ANCOVA, treatment x time interaction $p = 0.004$). However, change in total calcium was not different between the citrate-fasting and fortified-juice interventions at any time-point (Figure 5.4). As for ionised calcium, the results from the citrate-fasting and fortified-juice interventions were pooled in subsequent analyses (as citrate-juice; Figure 5.5).

Total calcium was increased from baseline between 1 and 6 hours after citrate-juice (all time-points $p < 0.0001$) and the dairy-meal (all $p < 0.02$) and between 2 and 6 hours after citrate-with-a-meal (all $p < 0.0001$). The maximal increase in total calcium after citrate-juice was 0.06 mmol/l, and was reached at 4 hours; after citrate with a meal was 0.08 mmol/l, and was reached at 6 hours; and after the dairy meal was 0.05 mmol/l, and was reached at 1 hour. Total calcium remained elevated from baseline 6 hours after citrate-juice by 0.04 mmol/l and after the dairy meal by 0.03 mmol/l. There were significant differences between the interventions in the change in total calcium (Figure 5.5). No participant had a total calcium measurement above the normal reference range (greater than 2.55 mmol/l) over 6 hours at any time-point. The AUCs for total calcium are presented in Figure 5.6, and were not different between the interventions.

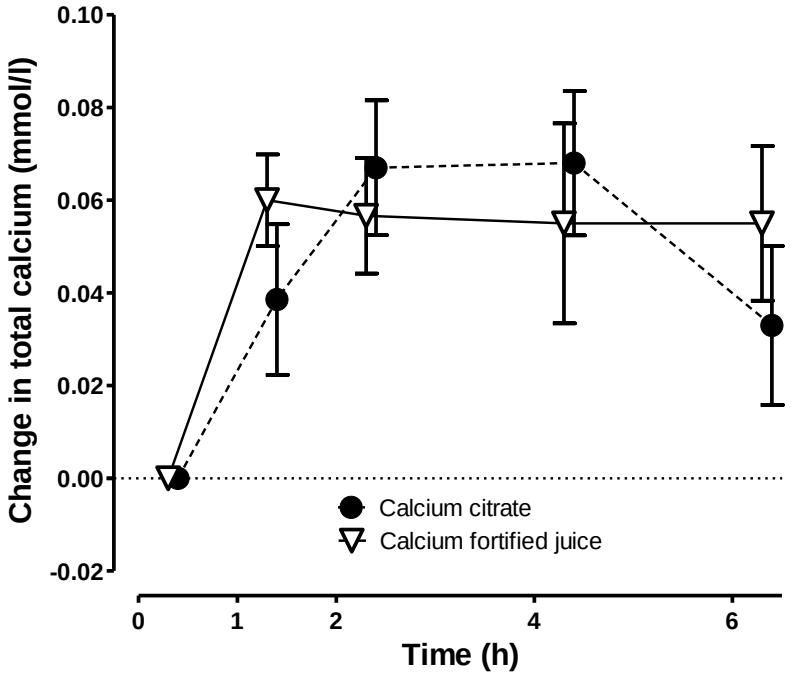

Figure 5.4 Change in serum total calcium over 6 hours in postmenopausal women (n = 10) after the ingestion of 500 mg of calcium citrate or 500 mg of calcium from fortified juice. Values are mean ± SEM.

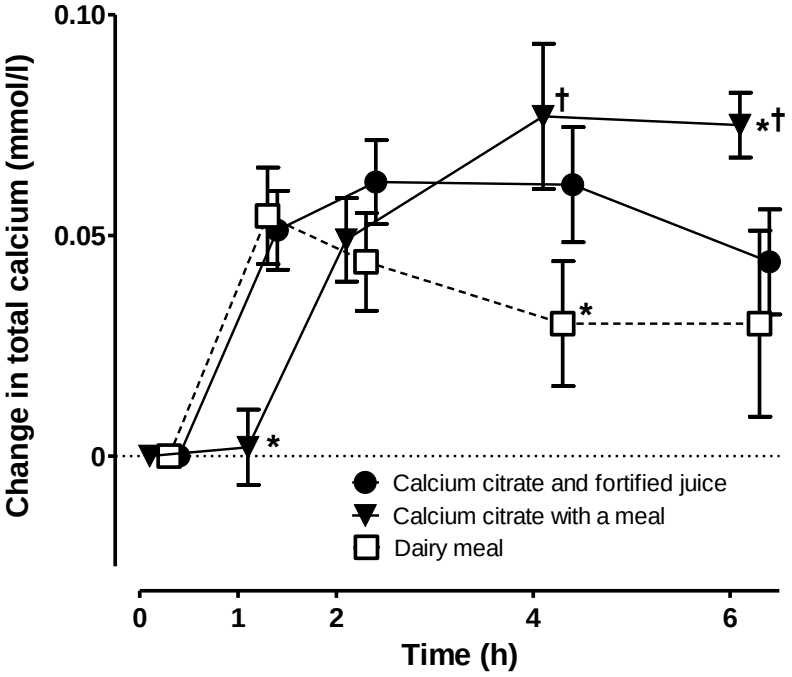

Figure 5.5 Change in serum total calcium over 6 hours in postmenopausal women (n = 10) after the ingestion of 500 mg of calcium citrate or fortified juice, 500 mg of calcium citrate with a meal, or 500 mg of calcium from a dairy meal. Values are mean ± SEM. There was a significant treatment x time interaction (ANCOVA, $p = 0.004$). * Significantly different from calcium citrate and fortified juice; † significantly different from the dairy meal, $p < 0.05$.

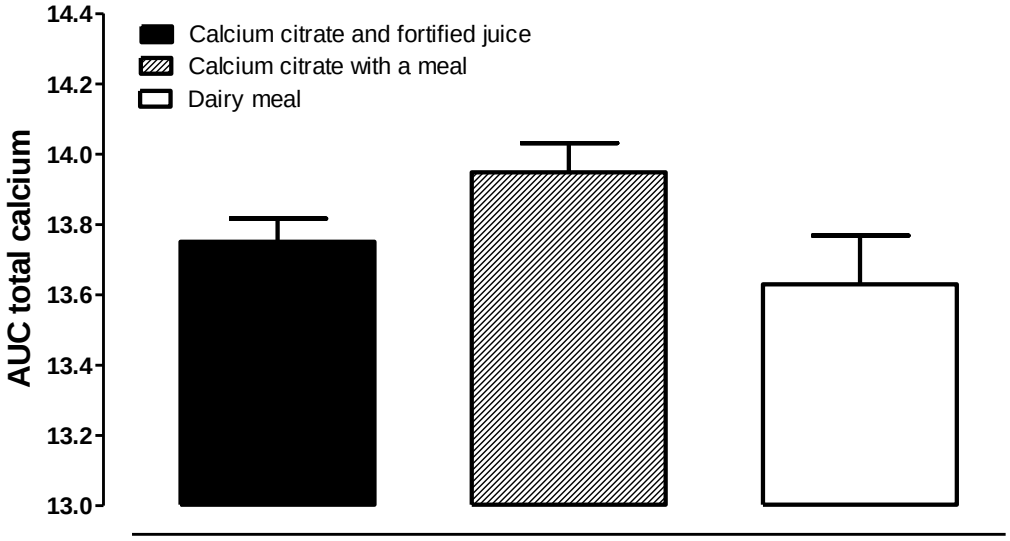

Figure 5.6 Area under the curve (AUC) of the change in serum total calcium in postmenopausal women over 6 hours after 500 mg of calcium citrate or fortified juice, 500 mg of calcium citrate with a meal or 500 mg of calcium from a dairy meal. The AUCs for total calcium were not significantly different between the interventions ($p = 0.08$).

Phosphate

There were significant differences in the change in phosphate between the four interventions (ANCOVA, treatment x time interaction $p < 0.0001$). There was a greater reduction in phosphate after fortified-juice compared with citrate-fasting at 4 hours (Figure 5.7). As previously, the citrate-fasting and fortified-juice interventions were pooled (Figure 5.8).

Phosphate was lower than baseline between 1 and 4 hours after citrate-juice (all time-points $p < 0.03$), was increased from baseline at 4 hours after citrate-with-a-meal ($p = 0.0003$), and was lower than baseline between 1 and 2 hours (all $p < 0.007$) and increased from baseline at 4 hours ($p = 0.0001$), after the dairy meal. The maximal reduction in phosphate after citrate-juice was -0.08 mmol/l, and was reached at 4 hours. The maximal increase in phosphate after citrate-with-a-meal was 0.09 mmol/l, and was reached at 4 hours. The maximal reduction in phosphate after the dairy-meal was -0.09 mmol/l and was reached at 2 hours, and the maximal increase was 0.09 mmol/l, and was reached at 4 hours. There were significant differences between the interventions in the change in phosphate (Figure 5.8). The AUCs of phosphate after each intervention are presented in Figure 5.9.

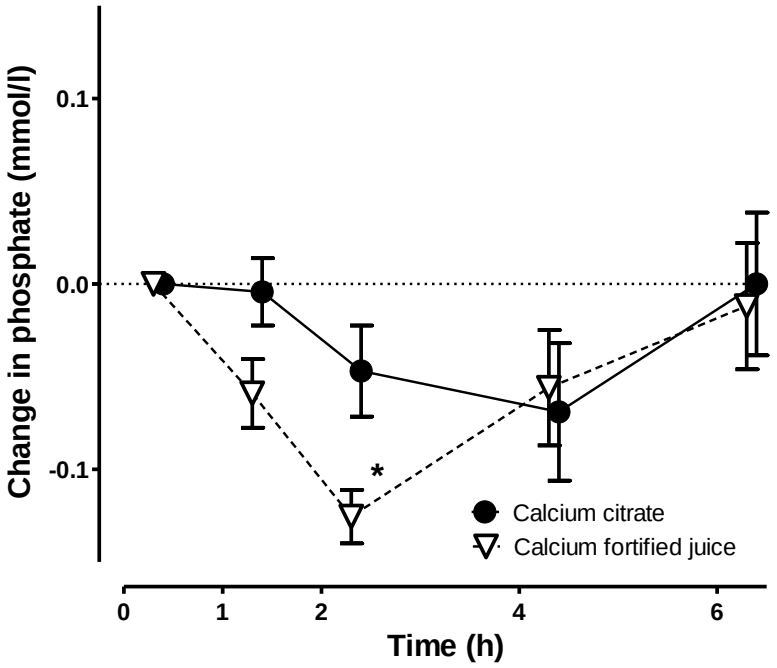

Figure 5.7 Change in serum phosphate over 6 hours in postmenopausal women (n = 10) after the ingestion of 500 mg calcium citrate or 500 mg of calcium from fortified juice. Values are mean ± SEM. * Significantly different from calcium citrate, $p < 0.05$.

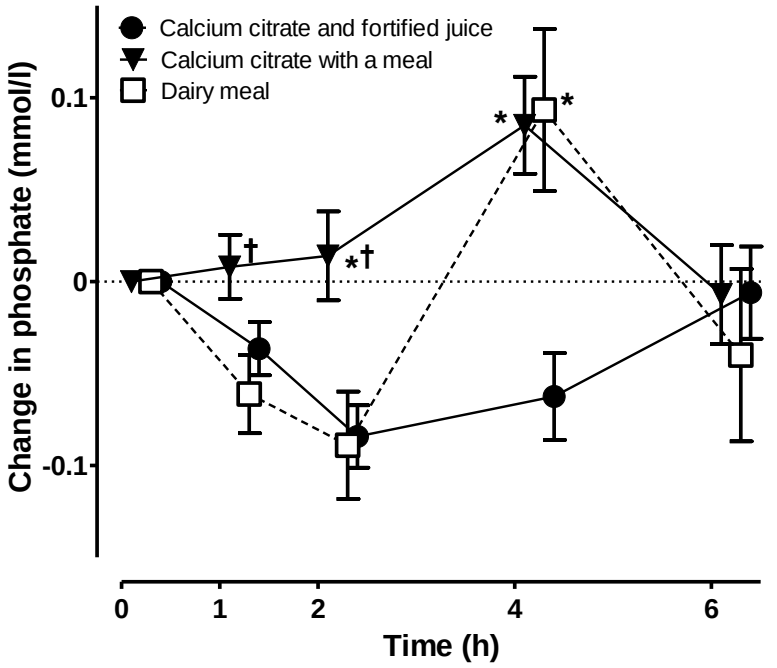

Figure 5.8 Change in serum phosphate over 6 hours in postmenopausal women (n = 10) after the ingestion of 500 mg of calcium citrate or fortified juice, 500 mg of calcium citrate with a meal and 500 mg of calcium from a dairy meal. Values are mean ± SEM. There was a significant treatment x time interaction (ANCOVA, $p < 0.0001$). * Significantly different from calcium citrate and fortified juice; † significantly different from the dairy meal, $p < 0.05$.

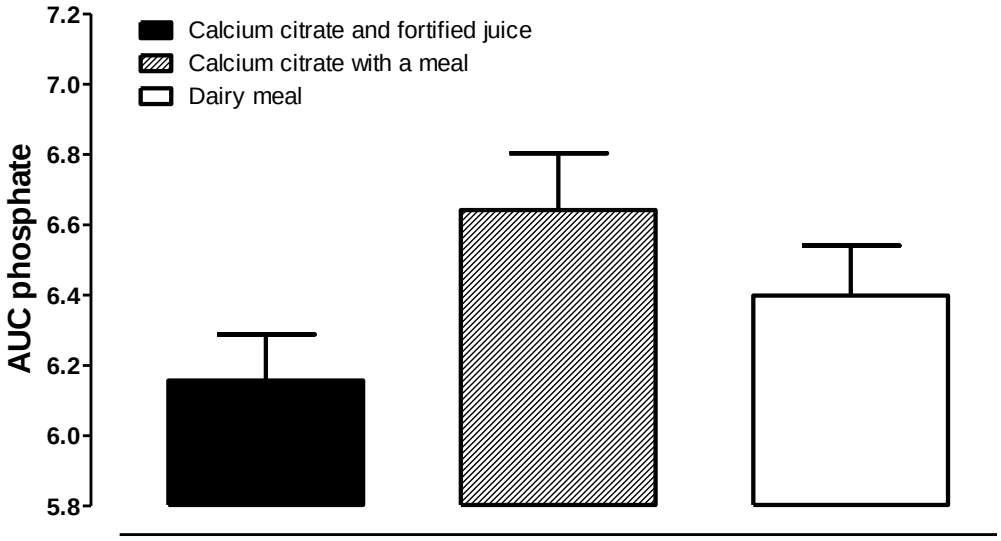

Figure 5.9 Area under the curve (AUC) of the change in serum phosphate in postmenopausal women over 6 hours after 500 mg of calcium citrate or fortified juice, 500 mg of calcium citrate with a meal or 500 mg of calcium from a dairy meal. The AUCs for phosphate were not significantly different between the sessions ($p = 0.07$).

Calcium-phosphate product

The time-course of the change in the calcium-phosphate product was examined with the results from the calcium-citrate and fortified-juice interventions pooled (Figure 5.10). The calcium-phosphate product was lower than baseline between 2 and 4 hours after citrate- juice (all time-points $p < 0.03$), was increased from baseline 4 hours after citrate-with-a-meal ($p < 0.0001$) and was lower than baseline between 1 and 2 hours ($p < 0.03$) and increased from baseline at 4 hours ($p < 0.0001$), after the dairy meal. The maximal reduction in the calcium-phosphate product after citrate-juice was -0.08 $mmol^2/l^2$ and was reached at 2 hours. The maximal increase in the calcium-phosphate product after citrate-with-a-meal was 0.12 $mmol^2/l^2$, and was reached at 4 hours. The maximal reduction in the calcium-phosphate product after the dairy meal was -0.10 $mmol^2/l^2$, and was reached at 2 hours, and the maximal increase was 0.12 $mmol^2/l^2$, and was reached at 4 hours. There were significant differences between the interventions in the change in calcium-phosphate product (Figure 5.10).

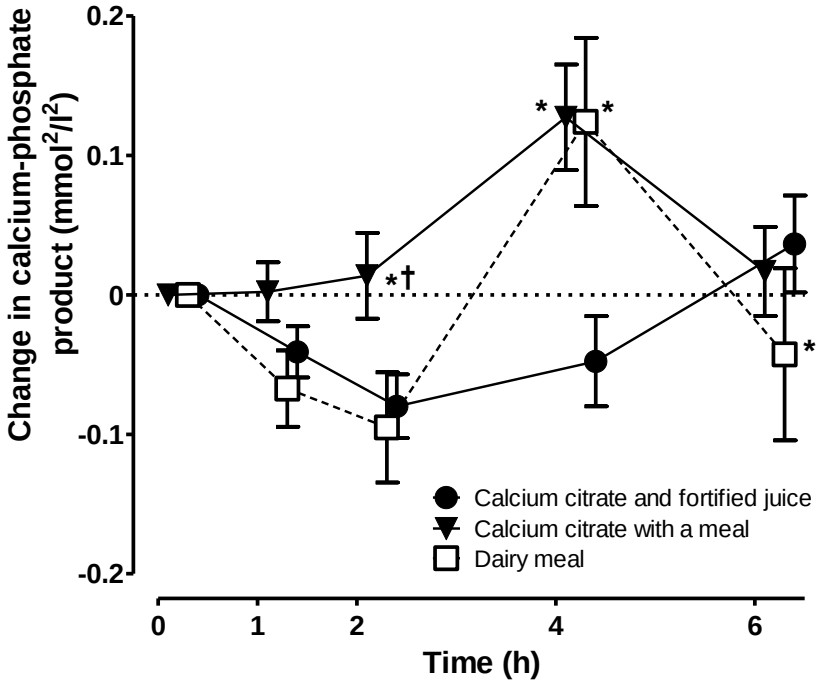

Figure 5.10 Change in serum calcium-phosphate product over 6 hours in postmenopausal women (n = 10) after the ingestion of 500 mg of calcium citrate or fortified juice, 500 mg of calcium citrate with a meal and 500 mg of calcium from a dairy meal. Values are mean ± SEM. There was a significant treatment x time interaction (ANCOVA, $p < 0.0001$). * Significantly different from calcium citrate and fortified juice; † significantly different from the dairy meal, $p < 0.05$.

5.4 DISCUSSION

In the present study, serum ionised and total calcium were significantly increased from baseline over 6 hours following the ingestion of 500 mg of calcium from all interventions. The elevations in ionised and total calcium after citrate-fasting and fortified-juice were identical, and were pooled as citrate-juice. Compared with citrate-juice, the increase in serum calcium was similar but delayed by 1-2 hours after the citrate-with-a-meal intervention, and was smaller at some time-points after the dairy-meal intervention.

Similar acute elevations in serum calcium have been previously reported following the ingestion of 400 – 500 mg of calcium from supplements [140, 141, 178, 279, 318] or fortified foods [141, 315]. The maximal mean increase in ionised and total calcium after 500 mg of calcium in the present study (0.03 mmol/l and 0.06 – 0.08 mmol/l, respectively) was smaller than that after 1000 mg of calcium in my previous trial (Chapter 3; 0.05 mmol/l and 0.09 mmol/l). However, the maximal increase in total calcium in the present study was still equivalent to one standard deviation of the baseline values (0.05 mmol/l). In a longitudinal study of postmenopausal women, Slinin et al [182] reported that a one standard deviation increase (0.09 mmol/l) in total calcium was associated with a 17% increase in the risk of a cardiovascular event. Similarly, Jorde et al [180] reported that a one standard deviation increase (0.10 mmol/l) in total calcium was associated with a 20% increase in the risk of myocardial infarction in men, and with a similar non-significant increase in women. Serum calcium remained elevated for up to 6 hours after the citrate-juice and citrate-with-a-meal interventions, suggesting two doses of 500 mg of supplemental calcium daily will result in some elevation in serum calcium for at least 12 hours/day. The elevation in serum calcium after fortified-juice might explain the reduction in bone loss in trials which have administered large doses of calcium-fortified foods [114, 115, 117, 118].

The present study is the first to examine the impact on serum calcium of a meal of dairy products, although others have reported changes following a single food. Karkkeinen et al reported that ionised calcium increased significantly after 400 mg of calcium from a supplement or cheese, but not after calcium from milk, sesame seeds or spinach [140]. Talbot et al [141] reported that 500 mg of calcium as unfortified milk resulted in a smaller increase in ionised calcium than calcium-fortified powdered milk, a calcium carbonate supplement or calcium-fortified yoghurt. Consistently, in the present study, the increase in serum calcium

was smaller at some time-points following the dairy-meal compared with the citrate-juice and citrate-with-a-meal interventions. In particular, ionised calcium appeared to peak 2 hours after the dairy-meal and decline thereafter, whereas ionised calcium was still increasing 6 hours after citrate-juice and citrate-with-a-meal. Although the differences between the dairy-meal and the other interventions were only small, in reality calcium from the diet will be consumed in doses less than 500 mg, spread over a day. The actual fluctuations in serum calcium associated with a high calcium diet are likely to be even smaller than that reported here.

The lesser calcaemic effect of dietary calcium in the present study and others [140, 141] could indicate that calcium is poorly absorbed from dietary sources. This is true for foods high in oxalic and phytic acid, which bind calcium and interfere with its absorption [142, 143]. In contrast, dairy calcium appears to be as well absorbed as calcium from supplements [319, 320]. The smaller calcaemic effect of dairy products might be due to protein and fat slowing gastric emptying and/or gastrointestinal transit time, and the rate at which calcium is released into the blood. However, in the present study, the citrate-with-a-meal intervention had a higher protein and fat content than the dairy-meal, but still resulted in a larger excursion in serum calcium. Therefore, the smaller calcaemic effect of dairy products must be related at least in part to other factors, such as the chemical form of calcium. For example, Green et al [321] observed a smaller increase in serum calcium following the ingestion of milk fortified with dairy calcium compared with milk fortified with calcium carbonate.

The increase in serum calcium was delayed by 1 - 2 hours after citrate-with-a-meal compared with citrate-juice. This likely reflects a delay in gastric emptying and/or gastrointestinal transit time after the meal. At 6 hours, total calcium was greater after citrate-with-a-meal compared with citrate-juice, suggesting calcium absorption was improved by the meal. Heaney has shown that the absorption of even highly soluble calcium salts is improved when they are taken with a meal [322]. In contrast to total calcium, ionised calcium was not different between the citrate-with-a-meal and citrate-juice interventions at 6 hours. These differences might be explained by increased phosphate after the meal, and the complexing of phosphate with ionised calcium. Importantly, as changes in ionised and total calcium were similar whether or not calcium was taken with a meal, this study does not support advice that the calcaemic effects of calcium supplements can be reduced by taking them with a meal.

The citrate-with-a-meal and dairy-meal interventions had a higher phosphate content than the citrate-juice intervention, and correspondingly resulted in an elevation in serum phosphate and calcium-phosphate product. Increased phosphate and calcium-phosphate product have been associated with cardiovascular risk in patients with CKD [323-325] and the general population [183, 299, 300]. However, high intakes of dairy products do not appear to increase cardiovascular risk [36, 326, 327]. Phosphate declined from baseline at 2 hours after citrate-juice and the dairy meal, but not after citrate-with-a-meal. This was probably due to the greater carbohydrate content of these meals, as carbohydrate after a fast results in the movement of phosphate into cells [298].

In summary, 500 mg of calcium as citrate, fortified juice or a dairy meal significantly elevated serum calcium from baseline over 6 hours. The elevations in serum calcium following calcium citrate and calcium-fortified juice were identical, suggesting fortified foods have a similar calcaemic effect to supplements, when taken in equivalent doses. This likely explains the reductions in bone loss in trials of calcium-fortified foods. Taking a calcium supplement after a meal containing protein and fat only delayed, but did not diminish, the elevation in serum calcium. Therefore, there is no evidence that the calcaemic effects of calcium supplements can be blunted by taking them with meals. Calcium obtained from a meal of dairy products resulted in a smaller elevation in serum calcium than calcium supplements and fortified juice. If the adverse cardiovascular risks associated with calcium supplements are mediated through their effects on serum calcium, then the differences in serum calcium observed in the present study might explain the different effects of supplemental and dietary calcium on cardiovascular risk.

CHAPTER 6: EFFECTS OF DIETARY CALCIUM INTAKE ON BONE MINERAL DENSITY AND FRACTURE RISK: A CROSS-SECTIONAL AND LONGITUDINAL ANALYSIS

6.1 INTRODUCTION

The increased risks of kidney stones [33, 35] and cardiovascular events [29] associated with calcium supplements have led to statements that the recommended intakes of calcium should be met through the diet [31, 32, 149]. Notably, in 2013, the United States Preventative Services Taskforce did not support the use calcium supplements [328]. However, the dietary calcium intakes of populations often fall below the recommended levels [22, 23, 63], and without the use of calcium supplements, increasing calcium intakes will be difficult. High dairy diets may not be acceptable to many people, and any lifestyle changes are notoriously difficult to maintain [329]. It is therefore imperative that efforts to increase dietary calcium intakes are based on evidence that this will benefit bone health.

In developing recommended intakes of calcium, the findings of calcium balance studies are heavily relied on [18, 28]. An analysis of early balance studies indicated that calcium intakes of 1500 mg/day were required for neutral calcium balance in postmenopausal women [10]. In a more recent analysis of calcium balance data, 740 mg/day was reported to be adequate for neutral calcium balance, regardless of age or sex [58]. As I described in Chapter 2, the precision of balance studies for estimating the relationship between calcium intake and long-term bone balance is unclear.

The positive effects of calcium supplements on bone loss and fracture risk are also considered when developing recommended intakes of calcium [18, 28]. However, most calcium supplement trials have administered doses of 1000 – 1200 mg/day of calcium to participants with dietary calcium intakes of 600 – 900 mg/day, which will have resulted in total calcium intakes which exceed the recommended levels. Thus, they cannot provide evidence that an intake of 1200 – 1300 mg/day is preferable to one of 400 – 1000 mg/day. For this reason, the findings of clinical trials of calcium supplements were not considered by the United Kingdom Committee on Medical Aspects of Food and Nutrition Policy when developing their recommended intakes of calcium [21].

The underlying premise in using the effects of calcium supplementation to predict what might occur with higher dietary calcium intakes seems to be that these effects relate to the correction of a dietary calcium deficiency. If true, it should follow that the effects of calcium supplements are greater in those with low dietary calcium intakes, who are calcium deficient, and smaller or non-existent in those with high intakes, who are calcium replete. Except for in one small trial [13], such an effect has not been demonstrated to date.

I have utilised an existing database from an RCT of calcium supplements to investigate the relationship between dietary calcium intake, BMD and fracture risk. Specifically, I have examined a) the relationship between dietary calcium intake and BMD, b) the relationship between dietary calcium intake and change in BMD and fracture risk and c) whether the effects of calcium supplementation on BMD and fracture risk are modified by dietary calcium intake.

6.2 METHODS

Participants

This study has previously been described in detail [137]. It was a randomised, placebo-controlled trial of 1471 healthy postmenopausal women, designed to assess the effects of calcium supplementation on BMD and fracture incidence. Participants were randomised to 1000 mg/day of calcium as citrate, or a placebo containing no calcium, for 5 years. Recruitment was by advertisements and by mail-outs using electoral rolls. Participants were aged over 55 years; more than 5 years post-menopause; had not been regular users of hormone replacement therapy, anabolic steroids, glucocorticoids, bisphosphonates, or calcium supplements in the previous year; were free of major systemic illnesses including untreated hypo- or hyperthyroidism, liver disease, malignancy or metabolic bone disease; and had serum creatinine <0.2 mmol/l and serum 25-hydroxyvitamin D >25 nmol/l. The lumbar spine BMD of participants was not below the age-appropriate normal range.

Data from the entire group of participants contributed to the baseline analysis of BMD; data from participants allocated to placebo contributed to the analysis of change in BMD and fracture risk over time; and data from participants allocated to calcium contributed to the

analysis of the effect of calcium supplements on BMD and fracture risk by dietary calcium intake.

Measurements

Spine (L1-4), total hip and total body scans were carried out at baseline, 30 months and 60 months, with a Lunar Expert dual-energy x-ray absorptiometer (GE-Lunar, Madison WI, software version 1.7). Serum 25-hydroxyvitamin D concentrations were measured at baseline by radioimmunoassay (Diasorin, Stillwater, MN) in a laboratory participating in the International Quality Assessment Scheme for Vitamin D Metabolites (DEQAS). Dietary calcium intake was assessed at baseline using a validated food frequency questionnaire [277]. This study received ethical approval from the local ethics committee and each subject gave written informed consent. The study was registered with the Australian Clinical Trials Registry, ACTRN 012605000242628.

Statistics

Data are summarised using mean and standard deviation, median and interquartile range or number (percentage and 95% confidence interval) as appropriate. Confidence intervals for proportions were calculated using www.openepi.com (accessed June 2014). Between quintile differences in variables with a normal distribution (whether baseline or change from baseline) were sought using one way analysis of variance (ANOVA, general linear modelling). Significant main effects were further investigated using the method of Tukey to preserve an overall 5% significance level within each test and tested for linear and higher order trends using orthogonal contrasts. For categorical data (i.e. current smoking or prevalent fracture) the null hypothesis of no difference in frequency between quintiles was tested using logistic regression. Unadjusted means (with 95% confidence intervals) are presented. Where indicated these analyses were repeated with covariate adjustment for age, weight, BMI, current smoking status and serum 25-hydroxyvitamin D.

Ordinary least squares linear regression was performed to examine the univariate association between BMD and dietary calcium and model both the slope and intercept. Data were plotted and residuals were inspected to assess the adequacy of the fit. Pearson's correlation coefficient was used to describe the linear association.

Multiple linear regression was performed to explore linear associations between BMD (as a continuous variable) and change in dietary calcium with adjustment for prespecified covariates. Colinearity diagnostics were inspected and the stability of the models assessed by comparing goodness of fit for models where the order of variables in the model was systematically varied. Goodness of fit for each final model was summarized by R-square statistic.

The time to first fracture was modelled using a proportional hazards approach (Cox) with and without adjustment for prespecified covariates using the lowest quintile of dietary calcium as the referent group. The assumption of proportional hazards was tested by including a quintile by time interaction effect in the model.

Since comparisons were pre-planned, no adjustment to the overall significance level (for multiplicity) was needed or employed. Unless where specified all analyses were performed using procedures of SAS v9.3 (SAS Institute Inc). All tests were two tailed and $P< 0.05$ was considered significant.

6.3 RESULTS

Dietary calcium intake and bone mineral density

The baseline characteristics of participants are presented in Table 6.1. The mean dietary calcium intake of the whole group was 860 mg/day, and the means in the first and fifth quintiles were 410 mg/day and 1450 mg/day, respectively. The only clinical variable significantly related to calcium intake was prevalent fracture. In post-hoc testing, prevalent fracture was significantly higher in quintile 5 than in quintiles 2, 3 and 4.

Total body, total hip and lumbar spine BMD by quintiles of dietary calcium intake are presented in Figure 6.1. BMD was not related to quintile of dietary calcium intake. Adjusting for age, weight, BMI, current smoking status and serum 25–hydroxyvitamin D did not change the relationship for total body BMD ($p = 0.09$), total hip BMD ($p = 0.18$) or lumbar spine BMD ($p = 0.25$).

Total body, total hip and lumbar spine BMD by individual values for dietary calcium intake are presented in Figure 6.2. Dietary calcium intake was significantly associated with total body and total hip BMD, and not significantly associated with lumbar spine BMD.

Multiple regression analyses using prespecified variables for total body, total hip and lumbar spine BMD are presented in Tables 6.2 – 6.4. Age, weight and dietary calcium were significant predictors of total body BMD. A 300 mg/day increase in dietary calcium intake was associated with a 0.005 g/cm^2 (0.5%) higher total body BMD. For total hip BMD, age, weight, height and dietary calcium intake were significant predictors, with a 300 mg/day increase in dietary calcium associated with a 0.006 g/cm^2 (0.7%) higher total hip BMD. Only weight was a significant predictor of lumbar spine BMD, although dietary calcium came close ($p = 0.08$). There was a trend for a 300 mg/day increase in dietary calcium intake to be associated with a 0.004 g/cm^2 (0.5%) higher lumbar spine BMD.

Table 6.1 Baseline characteristics of total group by quintiles of dietary calcium intake.

	Quintiles					Total	P
	1	**2**	**3**	**4**	**5**		
Quintile boundaries (mg/day calcium)	<546	546-705	706-884	885-1145	>1146	NA	NA
n	294	294	295	294	294	1471	NA
Dietary calcium intake (mg/day)	408 (110)	630 (44)	790 (51)	1005 (78)	1454 (290)	862 (390)	NA
Age (y)	74.0 (4.2)	74.2 (4.0)	74.1 (4.2)	74.1 (4.5)	73.8 (4.3)	74.0 (4.2)	0.73
Years since menopause	25.3 (6.8)	25.0 (5.7)	24.8 (6.1)	23.9 (6.9)	24.2 (6.2)	24.4 (6.4)	0.11
Weight (kg)	66.9 (11.1)	66.5 (10.9)	67.4 (11.9)	66.8 (11.2)	66.9 (11.3)	66.8 (11.1)	0.94
Height (cm)	158.4 (5.8)	159.0 (5.8)	159.0 (5.6)	159.2 (5.8)	159.5 (5.7)	158.9 (5.6)	0.27
Body mass index (kg/m^2)	26.6 (4.0)	26.3 (4.2)	26.6 (4.5)	26.4 (4.4)	26.2 (4.0)	26.5 (4.2)	0.69
Bone mineral density (g/cm^2)							
Total Body	1.02 (0.09)	1.03 (0.09)	1.04 (0.08)	1.04 (0.09)	1.04 (0.10)	1.03 (0.09)	0.1
Total Hip	0.87 (0.13)	0.85 (0.13)	0.86 (0.13)	0.86 (0.13)	0.87 (0.14)	0.86 (0.13)	0.21
Lumbar Spine	0.84 (0.15)	0.86 (0.13)	0.86 (0.11)	0.86 (0.12)	0.86 (0.16)	0.85 (00.14)	0.31
Bone density T-score							
Total Body	-1.3 (1.2)	-1.2 (1.1)	-1.1 (1.1)	-1.1 (1.1)	-1.1 (1.2)	-1.1 (1.2)	0.10
Total Hip	-1.3 (1.1)	-1.3 (1.1)	-1.2 (1.1)	-1.1 (1.1)	-1.1 (1.2)	-1.2 (1.1)	0.21
Lumbar Spine	-1.2 (1.5)	-1.0 (1.5)	-0.9 (1.5)	-1.0 (1.5)	-1.0 (1.5)	-1.0 (1.5)	0.2
Serum 25-hydroxyvitamin D (nmol/)	52.3 (19.0)	51.6 (18.2)	51.5 (19.3)	52.0 (20.0)	51.5 (19.0)	51.8 (19.1)	0.98
Current smokers % (95% CI)	4.8 (2.7, 7.7)	3.4 (1.7, 6.0)	2.7 (1.3, 5.1)	1.7 (0.6, 3.7)	2.4 (1.0, 4.7)	3.0 (2.2, 4.0)	0.24
Prevalent fracture % (95% CI)	31 (26, 36)	26 (21, 31)	22 (18, 27)	28 (23, 33)	36 (31, 42)	29 (27, 31)	**0.005**

CI = confidence interval. Data are mean (SD) unless stated otherwise. The P value is for one-way ANOVA across the quintiles

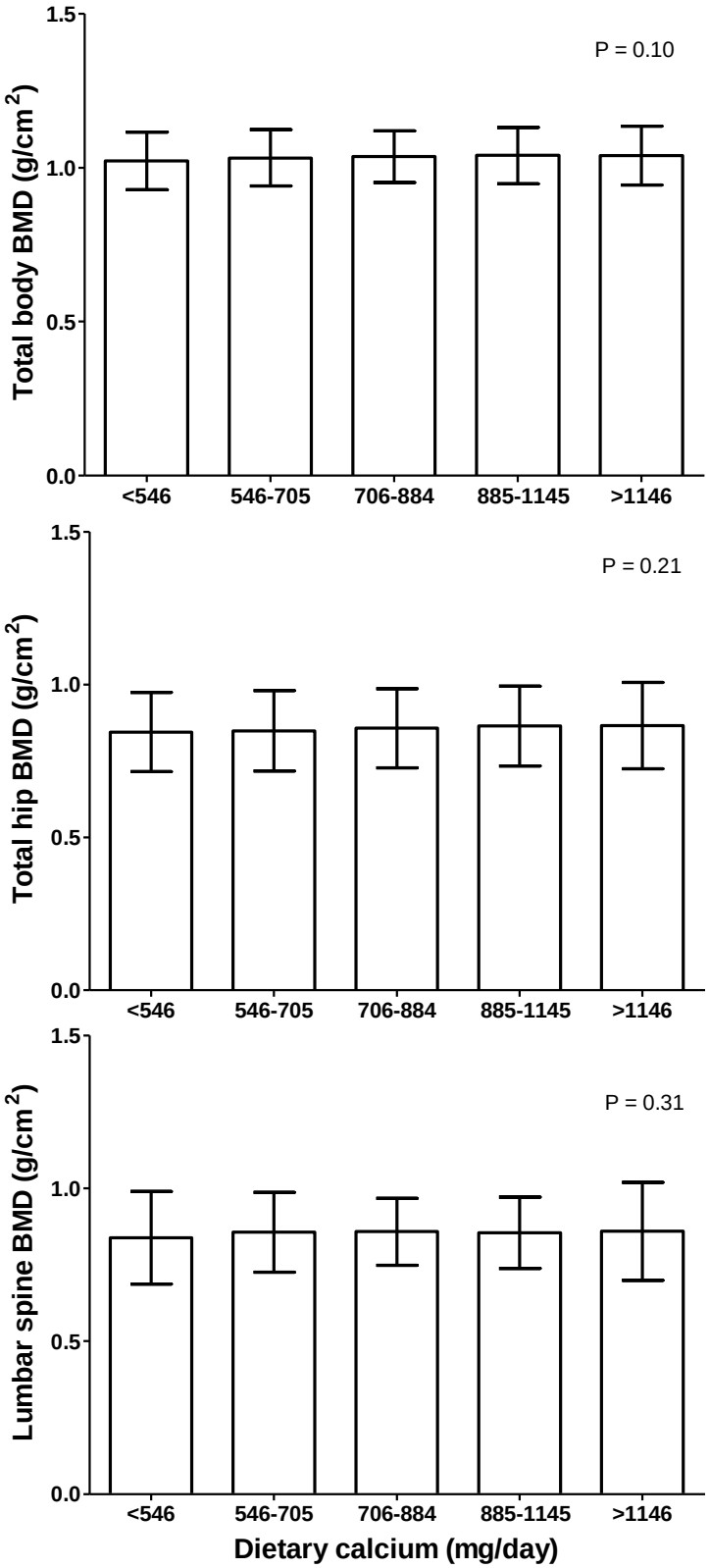

Figure 6.1 Total body, total hip and lumbar spine BMD in normal postmenopausal women by quintile of mean dietary calcium intake. There were no significant effects of quintile of dietary calcium intake on BMD. Data are mean ± 95% confidence intervals.

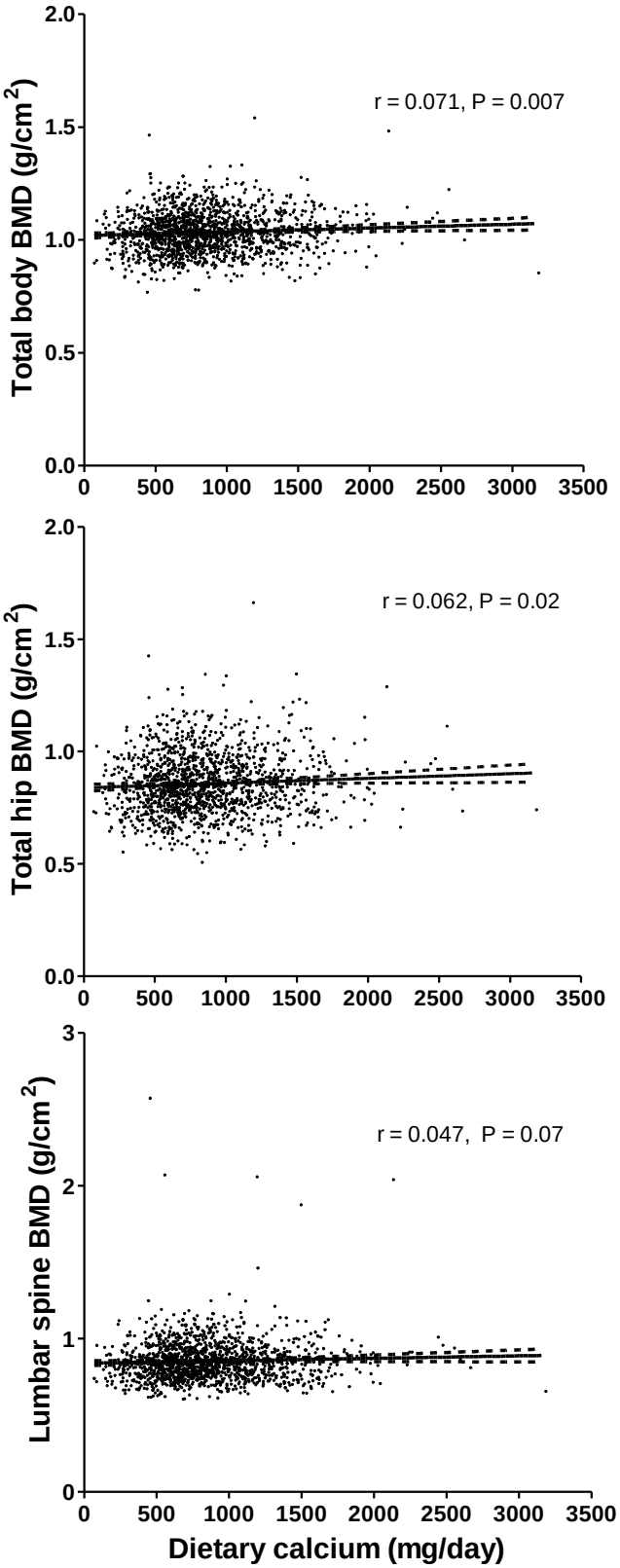

Figure 6.2 Total body, total hip and lumbar spine BMD by dietary calcium intake. The dark lines are the regression (with 95% confidence intervals) for this relationship.

Table 6.2 Multiple linear regression of prespecified variables and total body BMD.

Independent variable	**Unit**	**Regression coefficient**	**P**
P for model <0.0001, R^2 for model 0.21			
Age	year	-0.003	**<0.0001**
Weight	kg	0.003	**<0.0001**
Dietary calcium	300 mg/day	0.005	**0.004**
Height	cm	0.0002	0.67
Smoking status	Current (yes/no)	-0.02	0.15
Serum 25-hydroxyvitamin D	nmol/l	-0.00002	0.83

Table 6.3 Multiple linear regression of prespecified variables and total hip BMD.

Independent variable	**Unit**	**Regression coefficient**	**P**
P for model <0.0001, R^2 for model 0.20			
Age	year	-0.005	**<0.0001**
Weight	kg	0.003	**<0.0001**
Dietary calcium	300 mg/day	0.006	**0.01**
Height	cm	-0.002	**0.004**
Smoking status	Current (yes/no)	-0.02	0.19
Serum 25-hydroxyvitamin D	nmol/l	0.0003	0.07

Table 6.4 Multiple linear regression of prespecified variables and lumbar spine BMD.

Independent variable	**Unit**	**Regression coefficient**	**P**
P for model <0.0001, R^2 for model 0.19			
Age	year	-0.0002	0.83
Weight	kg	0.004	**<0.0001**
Dietary calcium	300 mg/day	0.004	0.08
Height	cm	-0.006	0.33
Smoking status	Current (yes/no)	-0.02	0.32
Serum 25-hydroxyvitamin D	nmol/l	-0.00005	0.76

Dietary calcium intake and change in bone mineral density and fracture risk

The baseline characteristics of participants in the group allocated to placebo are presented in Table 6.5. The mean dietary calcium intake of the total group was 850 mg/day, and the means in the first and fifth quintiles were 410 mg/day and 1450 mg/day, respectively. The only clinical variables related to dietary calcium intake were height and prevalent fracture. In post-hoc test, height was significantly higher in quintile 4 than in quintile 1. Prevalent fracture was significantly higher in quintile 5 than in quintiles 2 and 3.

In the total group, the mean change over 5 years in total body BMD was -0.03 g/cm^2, total hip BMD -0.03 g/cm^2 and in lumbar spine BMD -0.002 g/cm^2. Change in total body, total hip and lumbar spine BMD by quintiles of dietary calcium intake are presented in Figure 6.3. Changes in BMD were not related to quintile of dietary calcium intake. Adjusting for age, weight, height, current smoking status and serum 25–hydroxyvitamin D did not change the relationship for total body BMD ($p = 0.50$), total hip BMD ($p = 0.58$) or lumbar spine BMD ($p = 0.97$). Changes in BMD by individual values for dietary calcium intake are presented in Figure 6.4, and again, no relationship with change in BMD was observed.

Multiple regression analyses using prespecified variables for change in BMD are presented in Tables 6.6 – 6.8. None of the prespecified variables, including dietary calcium intake, was a significant predictor of change in total body BMD, although smoking status came close ($p = 0.06$). Similarly, none of the prespecified variables was a significant predictor of change in total hip BMD. Age and weight were both significant predictors of the change in lumbar spine BMD.

The HR for time to first fracture is presented in Figure 6.5. The HR was not different across quintiles of dietary calcium intake. Adjusting for age, weight, height, smoking status and 25-hydroxyvitamin D did not change this relationship ($p = 0.52$).

Table 6.5 Baseline characteristics of placebo treated group by quintile of mean dietary calcium intake

	Quintile					Total	P
	1	**2**	**3**	**4**	**5**		
Quintile boundaries (mg/day calcium)	<546	546-705	706-884	885-1145	>1146	NA	NA
n	151	150	141	150	147	739	NA
Dietary calcium intake (mg/day)	409 (112)	625 (45)	787 (49)	1006 (80)	1450 (255)	853 (381)	NA
Age (y)	73.8 (4.0)	74.2 (4.2)	74.4 (4.5)	74.2 (4.6)	73.7 (4.3)	74.3 (4.3)	0.64
Years since menopause	25.7 (6.8)	25.3 (5.7)	25.2 (6.3)	24.1 (6.8)	24.0 (6.0)	25.0 (6.3)	0.13
Weight (kg)	66.6 (10.8)	66.6 (11.6)	66.7 (11.3)	67.2 (11.1)	67.9 (12.4)	67.1 (11.8)	0.86
Height (cm)	158.2 (6.0)	158.8 (5.5)	158.8 (6.2)	160.3 (5.9)	159.9 (5.6)	159.2 (5.9)	**0.013**
Body mass index (kg/m^2)	26.6 (3.8)	26.4 (4.5)	26.4 (4.4)	26.2 (4.3)	26.4 (4.4)	26.4 (4.2)	0.94
Bone mineral density (g/cm^2)							
Total Body	1.024 (0.087)	1.023 (0.091)	1.034 (0.081)	1.033 (0.090)	1.045 (0.098)	1.03 (0.09)	0.22
Total Hip	0.85 (0.12)	0.84 (0.12)	0.86 (0.11)	0.85 (0.13)	0.88 (0.15)	0.85 (0.13)	0.093
Lumbar Spine	0.83 (0.12)	0.84 (0.10)	0.85 (0.10)	0.85 (0.11)	0.87 (0.18)	0.85 (0.12)	0.12
Bone density T-score							
Total Body	-1.3 (1.1)	-1.3 (1.1)	-1.1 (1.0)	-1.2 (1.1)	-1.0 (1.2)	-1.2 (1.1)	0.22
Total Hip	-1.3 (1.0)	-1.4 (1.0)	-1.1 (0.9)	-1.2 (1.1)	-1.0 (1.3)	-1.2 (1.1)	0.093
Lumbar Spine	-1.2 (1.6)	-1.1 (1.4)	-0.9 (1.3)	-1.1 (1.5)	-1.0 (1.6)	-1.1 (1.5)	0.12
Serum 25-hydroxyvitamin D (nmol/l)	51.0 (18.8)	52.3 (19.3)	51.2 (19.3)	54.0 (21.3)	50.8 (17.4)	51.9 (19.2)	0.6
Current smokers % (95% CI)	2.6 (0.8, 6.3)	3.3 (1.2, 7.2)	2.1 (0.5, 5.7)	2.0 (0.5, 5.3)	2.7 (0.9, 6.4)	2.6 (1.6, 3.9)	0.95
Prevalent fracture % (95% CI)	31 (24, 39)	24 (18, 31)	23 (16, 30)	30 (23, 38)	38 (31, 46)	29 (26, 32)	**0.031**

CI = confidence interval. Data are mean (SD) unless stated otherwise. The P value is for one-way ANOVA across the quintiles

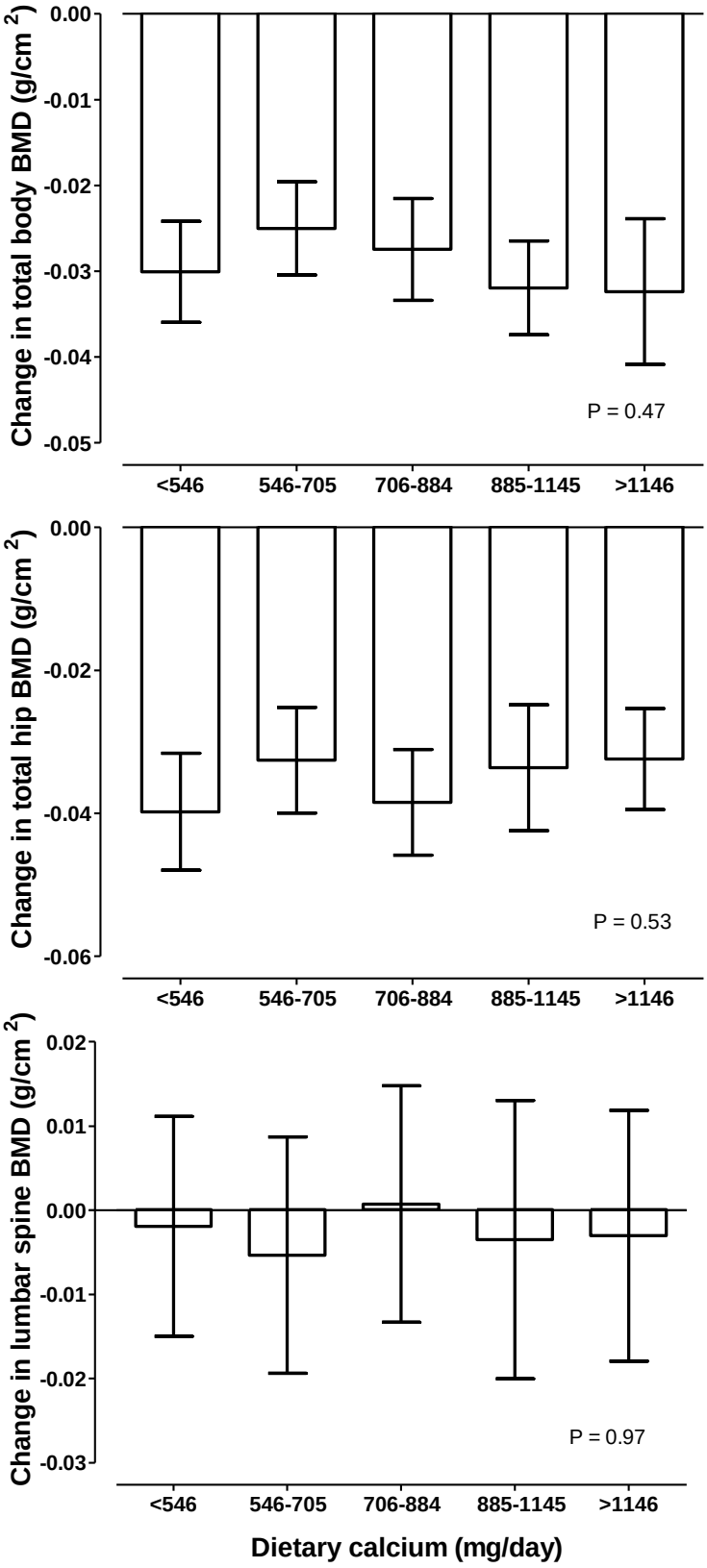

Figure 6.3 Change in total body, total hip and lumbar spine BMD over 5 years in normal postmenopausal women by quintile of mean dietary calcium intake. There were no significant effects of quintile of dietary calcium intake on change in in BMD. Data are mean ± 95% confidence intervals.

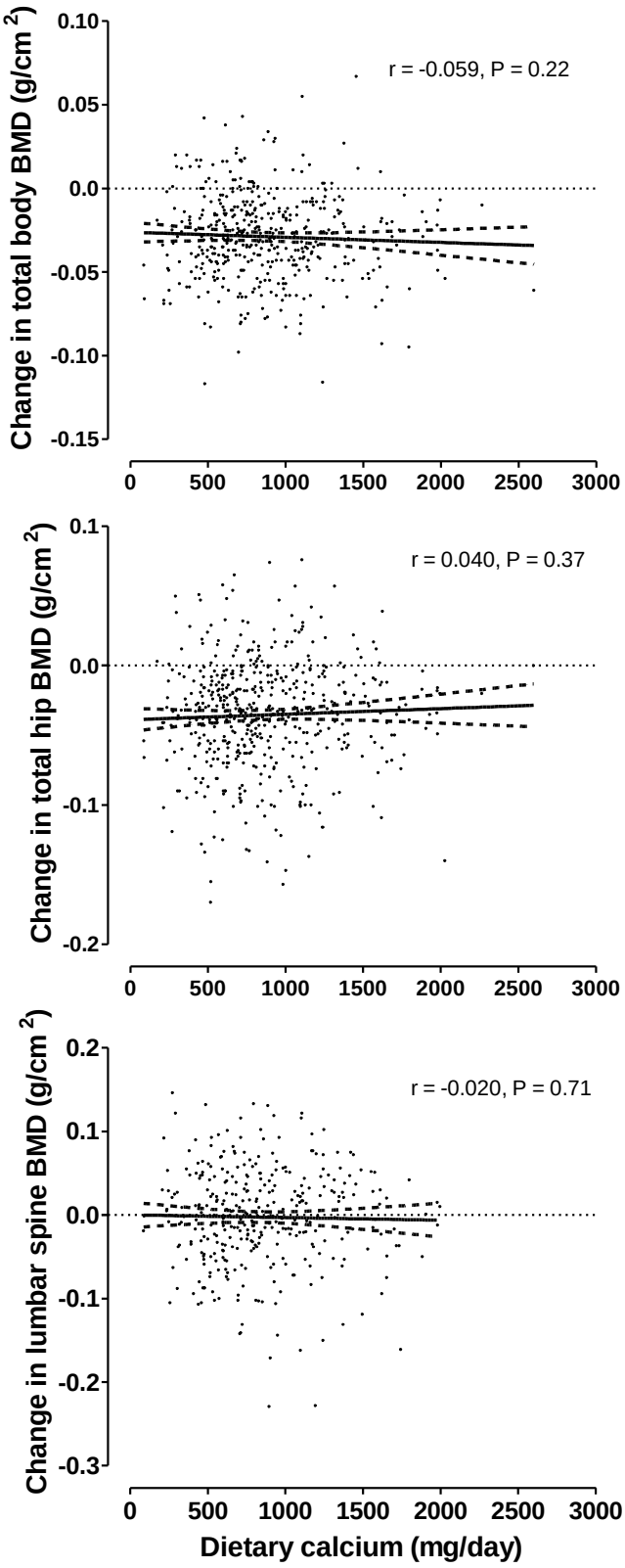

Figure 6.4 Change in total body, total hip and lumbar spine BMD over 5 years in normal postmenopausal women by dietary calcium intake. The dark lines are the regression (with 95% confidence intervals) for this relationship

Table 6.6 Multiple linear regression of prespecified variables and change in total body BMD.

Independent variable	**Unit**	**Regression coefficient**	**P**
P for model 0.32, R^2 for model 0.02			
Age	year	0.0003	0.41
Weight	kg	0.0002	0.47
Dietary calcium	300 mg/day	-0.001	0.33
Height	cm	-0.00004	0.88
Smoking status	Current (yes/no)	0.02	0.06
Serum 25-hydroxyvitamin D	nmol/l	-0.00003	0.73

Table 6.7 Multiple linear regression of prespecified variables and change in total hip BMD.

Independent variable	**Unit**	**Regression coefficient**	**P**
P for model 0.70, R^2 for model 0.008			
Age	year	-0.00007	0.87
Weight	kg	0.0005	0.18
Dietary calcium	300 mg/day	0.001	0.45
Height	cm	0.0003	0.36
Smoking status	Current (yes/no)	0.01	0.32
Serum 25-hydroxyvitamin D	nmol/l	-0.000026	0.78

Table 6.8 Multiple linear regression of prespecified variables and change in lumbar spine BMD.

Independent variable	**Unit**	**Regression coefficient**	**P**
P for model 0.0002, R^2 for model 0.08			
Age	year	0.003	**0.0001**
Weight	kg	0.002	**0.004**
Dietary calcium	300 mg/day	-0.002	0.36
Height	cm	0.0008	0.17
Smoking status	Current (yes/no)	0.03	0.15
Serum 25-hydroxyvitamin D	nmol/l	-0.0001	0.48

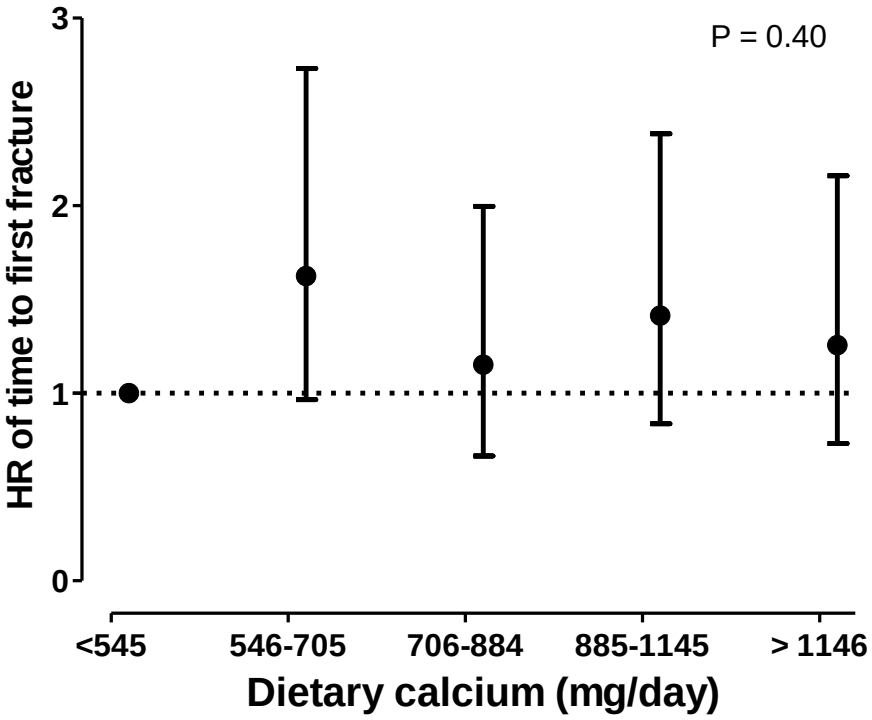

Figure 6.5 Hazard ratio (HR) of time to first fracture in normal postmenopausal women over 5 years by quintile of mean dietary calcium intake. There were no significant effects of quintile of dietary calcium intake on fracture risk. Data are HR ± 95% confidence intervals.

Effect of calcium supplementation on bone mineral density and fracture risk by dietary calcium intake

The baseline characteristics of participants allocated to calcium supplements are presented in Table 6.9. The mean dietary calcium intake of the calcium treated group was 860 mg/day, and the means in the first and fifth quintiles were 410 mg/day and 1460 mg/day, respectively. No clinical variables were related to dietary calcium intake.

Change in total body, total hip and lumbar spine BMD by treatment allocation and quintile of dietary calcium are presented in Figure 6.6. The changes in BMD in the placebo group are included for comparison. There was a significant effect of treatment allocation on change in BMD at all sites, as has been reported previously [137]. There was no effect of dietary calcium quintile on change in BMD at any site. There was no interaction between treatment allocation and quintile of dietary calcium intake on change in BMD at any site.

The HR of time to first fracture by treatment allocation and dietary calcium quintile are presented in Figure 6.7. There was no significant effect of treatment allocation on the time to first fracture (also reported previously [137]). There was similarly no effect of dietary calcium quintile on the time to first fracture, and no interaction between treatment allocation and dietary calcium quintile.

Table 6.9 Baseline characteristics of calcium treated group by quintile of mean dietary calcium intake.

	Quintiles					Total	P
	1	**2**	**3**	**4**	**5**		
Quintile boundaries (mg/day calcium)	<546	546-705	706-884	885-1145	>1146	NA	NA
n	143	144	154	144	147	732	NA
Dietary calcium intake (mg/day)	406 (109)	635 (43)	793 (54)	1004 (76)	1458 (321)	862 (390)	NA
Age (y)	74.2 (4.4)	74.3 (3.8)	73.7 (4.0)	74.1 (4.4)	73.8 (4.4)	74.0 (4.2)	0.75
Years since menopause	24.9 (6.8)	24.7 (5.7)	24.4 (5.9)	23.7 (6.9)	24.5 (6.4)	24.4 (6.4)	0.68
Weight (kg)	67.3 (11.4)	66.5 (10.2)	68.0 (12.4)	66.5 (11.4)	65.9 (10.1)	66.8 (11.1)	0.52
Height (cm)	158.7 (5.7)	159.2 (6.0)	159.2 (5.0)	158.1 (5.6)	159.1 (5.7)	158.9 (5.6)	0.4
Body mass index (kg/m^2)	26.7 (4.2)	26.2 (3.9)	26.8 (4.7)	26.7 (4.6)	26.0 (3.6)	26.5 (4.2)	0.45
Bone mineral density (g/cm^2)							
Total Body	1.02 (0.10)	1.04 (0.09)	1.04 (0.09)	1.05 (0.92)	1.03 (0.09)	1.04 (0.09)	0.12
Total Hip	0.84 (0.14)	0.86 (0.14)	0.85 (0.14)	0.87 (0.13)	0.86 (0.13)	0.86 (0.14)	0.33
Lumbar Spine	0.85 (0.18)	0.87 (0.15)	0.86 (0.12)	0.86 (0.13)	0.85 (0.14)	0.86 (0.14)	0.48
Bone density T-score							
Total Body	-1.3 (1.3)	-1.1 (1.2)	-1.1 (1.1)	-1.0 (1.2)	-1.1 (1.2)	-1.1 (1.2)	0.12
Total Hip	-1.3 (1.2)	-1.2 (1.2)	-1.2 (1.2)	-1.0 (1.1)	-1.2 (1.1)	-1.2 (1.1)	0.33
Lumbar Spine	-1.2 (1.5)	-0.8 (1.6)	-0.9 (1.6)	-0.90 (1.6)	-1.1 (1.4)	-1.0 (1.5)	0.14
Serum 25-hydroxyvitamin D (nmol/l)	53.7 (19.1)	50.8 (17.2)	51.8 (19.3)	49.9 (18.5)	52.2 (20.4)	51.7 (18.9)	0.52
Current smokers (%)	7.0 (3.6, 12.1)	3.5 (1.3, 7.5)	3.3 (1.2, 7.0)	1.4 (0.2, 4.5)	2.0 (0.5, 4.5)	3.4 (2.3, 4.9)	0.085
Prevalent fracture (%)	31 (24, 39)	28 (21, 35)	22 (16, 29)	26 (20, 34)	33 (25, 41)	28 (25, 31)	0.22

Data are mean (SD) unless stated otherwise. The P value is for one-way ANOVA across the quintiles.

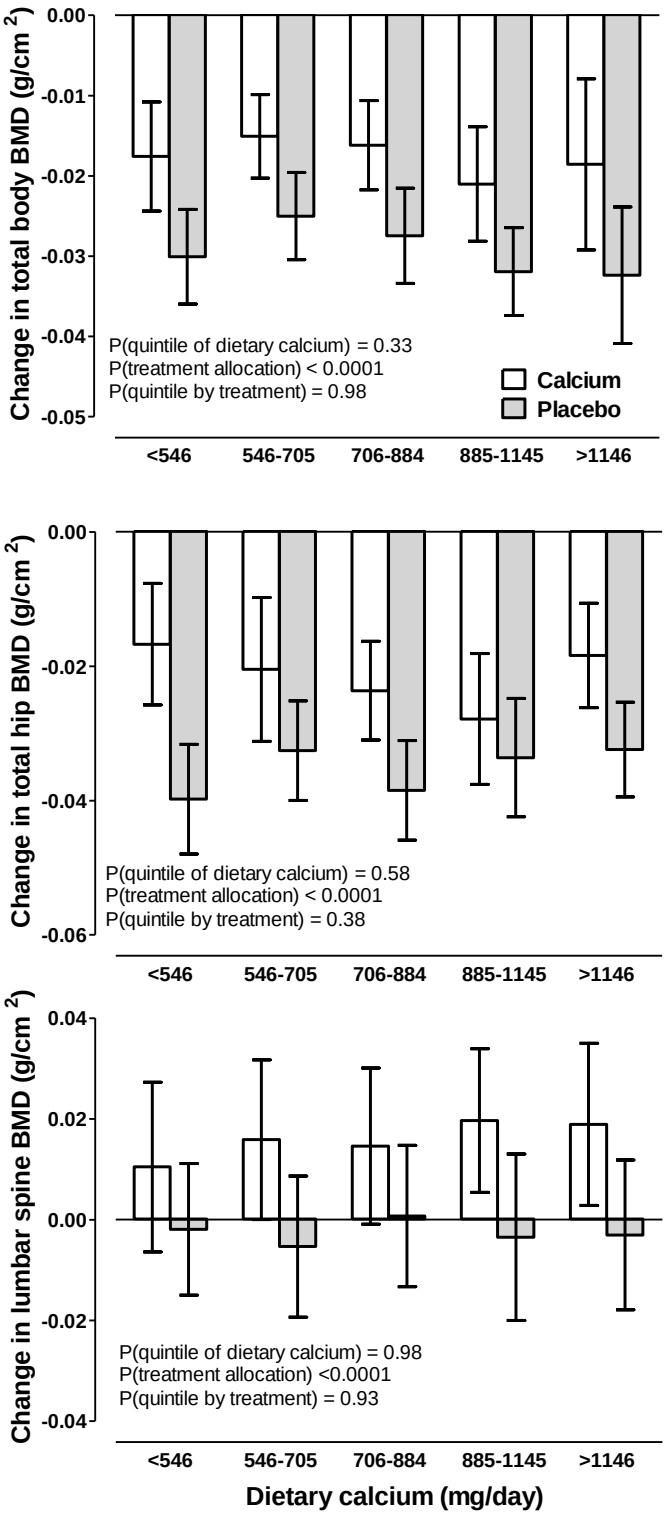

Figure 6.6 Change in total body, total hip and lumbar spine BMD over 5 years in normal postmenopausal women by treatment allocation (1000 mg/day of calcium or a placebo containing no calcium) and quintile of mean dietary calcium intake at baseline. There was a significant effect of treatment allocation and no effect of quintile of calcium intake on change in BMD, and no interaction between treatment allocation, quintile of calcium intake and change in BMD. Data are mean ± 95% confidence intervals.

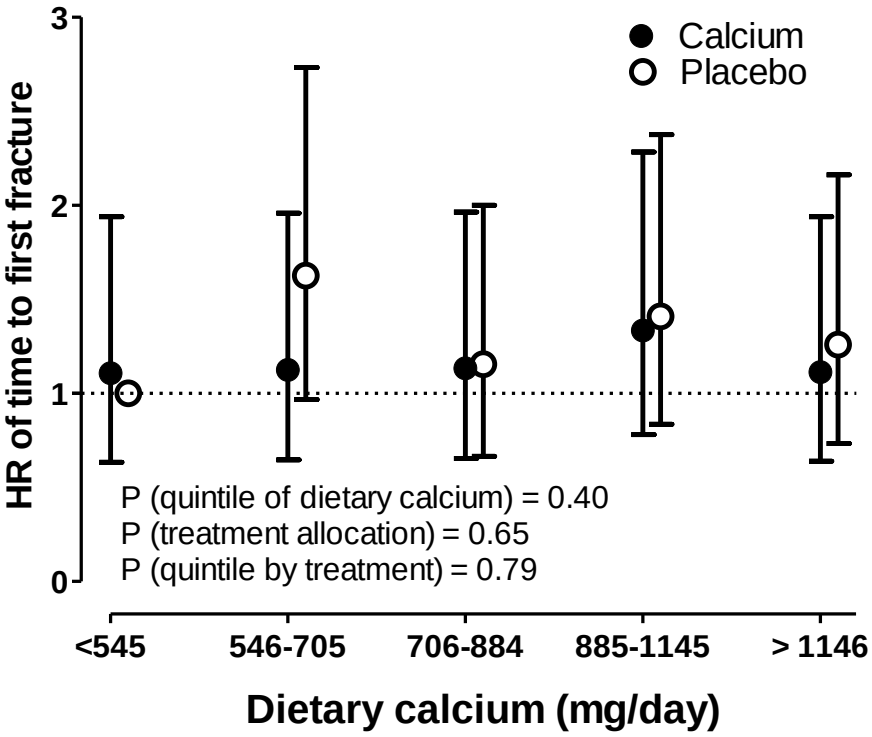

Figure 6.7 Hazard ratio (HR) of the time to first fracture over 5 years in normal postmenopausal women by treatment allocation (1000 mg/day of calcium or a placebo containing no calcium) and quintile of mean dietary calcium intake at baseline. There were no significant effects of treatment allocation or quintile of calcium intake on fracture risk. There was no significant interaction between treatment allocation, quintile of calcium intake and fracture risk. Data are mean ± 95% confidence intervals.

6.4 DISCUSSION

In the present study, dietary calcium intake was weakly associated with BMD at baseline, but was not associated with change in BMD or medium-term fracture risk. Furthermore, the effects of calcium supplementation on change in BMD and fracture risk were not modified by dietary calcium intake. These findings suggest that dietary calcium intake does not influence bone health in postmenopausal women, and that the effects of calcium supplements on bone health are not related to the correction of a dietary calcium deficiency.

A null relationship between dietary calcium intake and bone loss in adults is observed in most prospective cohort studies [83-89]. In contrast, some studies have reported an association between dietary calcium intake and bone loss; however, they have been small [96], have only reported increased bone loss at very low calcium intakes (less than 400 mg/day) [93, 96], or have reported an association in men but not women [94] or in never smokers but not ever smokers [95]. Consistent with a null relationship with bone loss, dietary calcium intake was not associated with fracture risk over 5 years in the present study. Most previous prospective cohort studies [38, 40, 114, 124-129, 330] and two meta-analyses of these studies (one which examined milk intake) [72, 132] have similarly reported no relationship between dietary calcium and fracture risk. One large study in postmenopausal women did find an increased risk of total and hip fracture in the lowest quintile of dietary calcium (less than 750 mg/day) compared with the third quintile (880 – 1000 mg/day), but no further reduction in total fracture risk, and an increase in hip fracture risk in the fifth quintile (more than 1100 mg/day) [77]. At baseline in the present study, a greater percentage of participants in the highest quintile of dietary calcium intake had a history of fracture than in some lower quintiles; however, this could be explained by individuals increasing their calcium intake after a fracture event. Collectively, there is little evidence that dietary calcium intake within the normal range in the diet influences bone loss and fracture risk, and importantly, no evidence that intakes of more than 1000 mg/day are preferable to 400 – 1000 mg/day.

In the present study there was a weak association between dietary calcium intake and BMD at baseline, the size of which was similar to that in several other cross-sectional studies. In the present study, a 300 mg/day increase in dietary calcium was associated with a 0.5 - 0.7% higher total body, total hip and lumbar spine BMD (although the effect on lumbar spine BMD did not reach significance). Similarly, in older women, a 400 mg/day increase in dietary

calcium was associated with a 0.7% higher distal radius BMD [99] and a 100 mg/day increase was associated with a 0.2% higher femoral neck BMD [97]. In older men, a 390 mg/day increase in dietary calcium was associated with a 0.6% higher femoral neck and 0.4% higher lumbar spine BMD [98]. In contrast, in a recent study, a trend for a positive relationship between dietary calcium intake and BMD was observed in men aged over 70 years, but an inverse relationship in women [79]. In another study, an association between dietary calcium and BMD was only observed among women who were vitamin-D deficient but not among those who were vitamin-D replete [107]. Thus, while a weak relationship between dietary calcium intake and absolute BMD is possible, the evidence is not consistent. The clinical relevance of this difference in BMD is also questionable. Based on FRAX [75], a 70 year old woman (weight 70 kg and height 165 cm) with a femoral neck BMD of 0.90 g/cm^2 (T-score -1.0) has a 10-year probability of a major osteoporotic fracture of 8.2% and hip fracture of 0.9%. The same woman with a dietary calcium intake 600 mg/day higher, and therefore with a BMD 1.0% higher, would have a 10-year probably of a major osteoporotic fracture of 8.0% and hip fracture of 0.8%. In the same woman with a BMD of 0.70 g/cm^2 (T-score -2.4), a difference in BMD of 1.0% would not influence the probability of a fracture.

The lack of an effect of dietary calcium intake on bone loss but small association with absolute BMD might suggest dietary calcium intake influences bone mass during a different stage in life, perhaps during the attainment of skeletal mass or the period of rapid bone loss following menopause. In a study in perimenopausal women, energy-adjusted dietary calcium intake was inversely associated with femoral neck, but not lumbar spine, bone loss [92]. Alternatively, the small association between dietary calcium intake and baseline BMD may have been a spurious finding. Individuals with high dietary calcium intakes are different from those with low intakes in a number of health-related behaviours such as physical activity, alcohol consumption and smoking status [36, 77, 78], and are more likely to report good health, be better educated and more prosperous [79]. High calcium intakes are also usually reflective of high dairy intakes and higher intakes of energy, protein, vitamin D and vitamin A [80]. Thus, these confounding factors may have contributed to an apparent effect.

The findings of the present study and others are in conflict with the findings of early calcium balance studies, which suggested intakes of 1500 mg/day were required for neutral balance in early postmenopausal women [10]. At intakes below this level, the obligatory losses of calcium were suggested to be greater than that absorbed, resulting in bone loss and negative calcium balance. However, in the present study, postmenopausal women with dietary calcium

intakes similar to that suggested to result in neutral calcium balance (1450 mg/day) lost as much bone over 5 years as women with intakes 1000 mg/day lower. It is well known that calcium balance will be slightly negative in older adults, due to age- and menopause-related bone loss. The findings of the present study suggest that the degree of negative calcium balance is not influenced by dietary calcium intake. This is consistent with a more recent analysis of calcium balance data by Hunt and Johnson [58], who predicted a neutral calcium balance at 740 mg/day for both men and women, but noted a tight control of balance from 400 to 1700 mg/day. Thus, the lack of a relationship between dietary calcium intake and bone loss may be explained by 'adaption' to the prevailing calcium intake, i.e. a greater amount of calcium may be absorbed and/or less calcium excreted at lower versus higher calcium intakes.

Calcium supplements have been demonstrated to reduce bone loss and possibly fracture risk to a modest degree [37], and these effects are often assumed to involve the correction of a dietary calcium deficiency [16]. High dietary calcium intakes and calcium supplementation have therefore been recommended interchangeably. However, in the present analysis, the reduction in bone loss associated with calcium supplementation was independent of baseline dietary calcium intake. This finding is consistent with subgroup analyses in most other calcium supplementation trials [62, 331-333], although one small trial reported an effect of calcium supplements only among those with dietary calcium intakes below 400 mg/day [13]. Since the effects of calcium supplements appear to be equivalent in those with high and low dietary intakes, this argues against their effects being related to the correction of a deficiency of calcium (unless a dietary calcium intake of ~1450 mg/day is considered deficient).

How then might the differences between calcium supplementation and high intakes of dietary calcium on bone loss and fracture risk be explained? Firstly, total calcium intake will in general be higher with calcium supplementation than when calcium is obtained from the diet. However, this does not fully explain these differences, since in the present study, those in the lowest quintile of dietary calcium who received a calcium supplement appeared to suffer less bone loss than those in the highest quintile of dietary calcium, yet both had similar total intakes of calcium (~1500 mg/day). Calcium supplements are likely to result in greater fluctuations in serum calcium than dietary calcium, and may therefore result in greater suppression of PTH and bone resorption. This is because calcium from supplements is taken in large boluses of 500 – 1000 mg, which as I demonstrated in Chapters 3 and 5, elevate serum calcium for at least 6 - 8 hours. Dietary calcium will be consumed in smaller doses

spread over a day, and even large doses of dietary calcium have a smaller impact on serum calcium than supplements (Chapter 5, [140, 141]). The *change* in calcium intake might also be important. Calcium supplementation results in an abrupt increase in calcium intake of 1000 mg/day, while dietary calcium intakes may remain more stable over time (or least not increase by and be maintained at +1000 mg/day). It was early recognised that adaption to a large increase in calcium intake is reflected by a period of positive calcium balance, during which calcium is accreted into bone, which ceases when equilibrium at the higher intake is achieved [7]. Correspondingly, the effects of calcium supplements on BMD are greatest in the 1 – 3 years after supplementation is initiated and minimal or null thereafter [14, 137]. Finally, calcium supplementation results in a large increase in an isolated nutrient, while calcium from the diet is consumed alongside a range of nutrients such as protein and phosphate [80, 81], which may blunt or otherwise modify the effects of dietary calcium on calcium and bone metabolism.

Strengths of the present study were its prospective design with both BMD and fracture outcomes, and the ability to adjust for a number of covariates. A limitation of this study may have been the method used to measure dietary calcium intake. Food frequency questionnaires can overestimate calcium intakes [334], and the intakes in this study may therefore be exaggerated. Nonetheless, a recent review concluded that food frequency questionnaires were a valid method for measuring nutrient intakes, particularly of calcium [335]. The findings of this study may not apply to men, or different ethnic or age groups.

In summary, the findings of the present study, and others, indicate that for postmenopausal women consuming more than 400 – 500 mg/day of calcium; dietary calcium intake will have little influence on bone loss and fracture risk. Recommendations to improve the bone health of populations should be evidence-based, and there is little, if any, evidence that meeting the recommended intakes of dietary calcium for older adults will benefit bone health. Encouraging individuals to increase dietary calcium could result in them being less likely to utilise interventions with proven anti-fracture efficacy, as they may believe they are already acting to reduce fracture risk. While calcium supplements have some small beneficial effects on bone loss and fracture risk, these effects do not appear to be related to the correction of dietary calcium deficiency.

CHAPTER 7: CALCIUM SUPPLEMENTS AND CANCER RISK: A META-ANALYSIS OF RANDOMISED CONTROLLLED TRIALS

7.1 INTRODUCTION

In addition to its suggested effects on bone and cardiovascular health, it is possible that calcium intake impacts on cancer risk. In observational studies, high calcium intakes have been associated with a reduced risk of colorectal [252-254] and breast cancer [255, 256], and an increased risk of prostate cancer [257-259]. However, these findings have not been consistent [260-265]. To-date, few RCTs of calcium supplements with or without vitamin D have reported cancer outcomes. In a 4 year fracture prevention trial, Lappe et al [266] reported calcium monotherapy decreased total cancer risk by 47% ($p = 0.06$), and calcium with vitamin D by 60% ($p = 0.01$). In contrast, three RCTs [270-272, 336] of calcium with or without vitamin D have found no evidence of an effect on cancer risk. Bolland et al reported no effect of calcium monotherapy on total cancer incidence in a 5 year trial in postmenopausal women [336]. The RECORD investigators found no effect of calcium with or without vitamin D on cancer mortality or incidence in older people treated for a median of 45 months [270]. The WHI investigators reported no effect of calcium with vitamin D on the risk of colorectal [271] or breast [272] cancer in over 36,000 postmenopausal women treated for an average of 7 years. However, in a recent re-analysis of the publicly available WHI dataset, significant interactions between treatment allocation, personal calcium or vitamin D supplement use and the risk of total, breast and colorectal cancers were found [30]. This suggested that widespread personal non-protocol supplement use may have obscured an effect of trial calcium with vitamin D allocation on cancer endpoints. When analyses were restricted to participants not taking personal calcium or vitamin D at baseline, calcium with vitamin D allocation significantly reduced the risk of total and breast cancer by 14 to 20%, and non-significantly reduced the risk of colorectal cancer by 17%.

Thus, there is some evidence that calcium and vitamin D supplements might lower cancer risk, but it remains uncertain whether these possible effects are related to calcium, vitamin D or the combination of both agents. I have updated a large database of randomised clinical trials of calcium supplements in older adults, originally assembled to assess the effect of

calcium supplements on cardiovascular risk, to determine whether calcium used as monotherapy impacts on cancer risk.

7.2 METHODS

In November 2007 Medline, Embase, and Cochrane Central were searched for randomised placebo-controlled trials of calcium supplements, using the terms "calcium", "randomised controlled trial", and "placebo" as text words, and corresponding MeSH terms [164]. The reference lists of meta-analyses published between 1990 and 2007 of the effect of calcium supplements on fracture, bone density, colorectal neoplasia, and blood pressure, and two clinical trial registries (ClinicalTrials.gov and Australian New Zealand Clinical Trial Registry) were searched. No language restrictions were applied. In February 2012, searches of the electronic databases were updated (Medline: 1966-February 2012, Embase: 1980-February 2012, Cochrane Central: January 2012).

Study selection

Studies were included if they were randomised, double-blind, placebo-controlled trials; the mean age of participants at baseline was more than 40 years; a dose of at least 500 mg/day of calcium was administered; at least 100 participants were randomised; participants of either sex were studied; and the duration of the trial was greater than 1 year. Studies were excluded if calcium and vitamin D were co-administered and compared with placebo (studies were eligible if vitamin D was given to both intervention and control groups); if calcium was administered in the form of a complex nutritional supplement or as a dietary modification; and if most participants had a major systemic disease other than osteoporosis or colorectal neoplasia.

Search results

Two investigators carried out the search and two investigators independently reviewed all potential studies to determine the adequacy of randomisation, concealment of allocation, blinding of participants and investigators and extent of loss to follow-up.

Data description

The lead author of each eligible trial was invited to provide patient-level data on cancer events that occurred during the study. When such data were not available, trial-level summary data was requested. Complete trial-level data were available on total cancer events for seven studies (9,447 participants) [14, 62, 137, 266, 267, 333, 337], on colorectal cancer for eight studies (9,863 participants) [14, 62, 137, 266-268, 333, 337], on breast cancer for six studies (7,641 participants) [14, 137, 266, 267, 333, 337], on prostate cancer events for three studies (1,806 participants) [62, 267, 333], and on cancer-related mortality for six studies (8,109 participants) [14, 62, 137, 267, 268, 333]. Partially complete trial-level data were available on total cancer for a further three studies (1,049 participants) [136, 268, 294], and on colorectal and breast cancer for a further two studies (633 participants) [136, 294]. No data on cancer events were available for six studies (2,743 participants) [13, 16, 332, 338-340].

Complete trial-level data were therefore available on total cancer events for 71% of participants, at least partially complete trial-level data for 79% of participants and no data for 21% of participants, from 16 eligible trials.

Ascertainment of cancer events

For seven studies, data on cancer events were supplied by the lead authors. Data were obtained from a combination of self-reports, hospital discharge data and death certificates [14, 62, 136, 137, 294, 333, 337] and cancer registries [333]. For one study, data on prostate cancer [269] and colorectal cancer [267] were obtained from published data, and data on total cancer, breast cancer, and cancer mortality were supplied by the lead author and derived from hospital discharge data and death certificates. For another study, data on colorectal cancer were obtained from published data [268], and data on cancer-related mortality were supplied by the lead author and derived from physician-reported cause of death. For the final study, data on total, breast and colorectal cancer were obtained from published data [266]. Cancers described as “skin”, “epidermal/epidermoid” or “basal cell” or the ICD code C44 were not considered in these analyses.

Endpoints

The prespecified primary end point was the incidence of first total cancer, excluding non-melanoma skin cancers. Secondary end points were the incidence of colorectal cancer, breast cancer, prostate cancer, and cancer-related mortality.

Statistical analysis

Statistical heterogeneity between summary data at trial level was assessed using Cochran's Q statistic ($P < 0.10$) and the I^2 statistic ($I^2 > 50\%$). No significant statistical heterogeneity existed between trials in any of the analyses. Random effects models were used to pool trial-level summary data. Publication bias was assessed using funnel plots. Analyses were done using SAS version 9.2 or Review Manager 5.1 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011). All tests were two-tailed and $P<0.05$ was considered significant.

In trials with patient-level data, each endpoint was analysed using a Cox's proportional hazards model stratified by study, and the HR and 95%CI reported. The assumption of proportional hazards was explored graphically and by carrying out a test for proportionality of the interaction between variables included in the model and the logarithm of time. Assessment of the effect modifying influence of covariates on outcomes was done by repeating the models including the following co-variates potentially associated with cancer incidence: age, sex, smoking status, BMI and weight; and by undertaking subgroup analyses using interaction terms between treatment allocation and the following pre-specified subgroups: sex, age (≥75 or <75 years), dietary calcium (above or below the median), serum 25-hydroxyvitamin D (≥50nmol/l or <50nmol/l) and supplement type (citrate, carbonate or lactogluconate-carbonate), where data were available for >80% of participants.
Based on the assumption that cancers diagnosed early on in the trials may have been present, but undetected, at baseline, we repeated these models including latent periods of 1 year, 2 years and censoring the first 50% of events. As a sensitivity analysis, the models were repeated including only trials in which data were obtained from unverified sources (self-reports) or verified sources (cancer registries).

7.3 RESULTS

The results of the literature search are shown in Figure 7.1, and the characteristics of the eligible studies in Table 7.1. All ten eligible studies providing data were randomised, double-blind, placebo-controlled trials. The quality of nine trials was reviewed in a previous meta-analysis [164]. One further study [136] did not describe the method of randomisation or allocation concealment, but reported details of participants who withdrew or were lost to follow-up, and compliance in ~90% of participants. Table 7.2 shows selected baseline characteristics of participants.

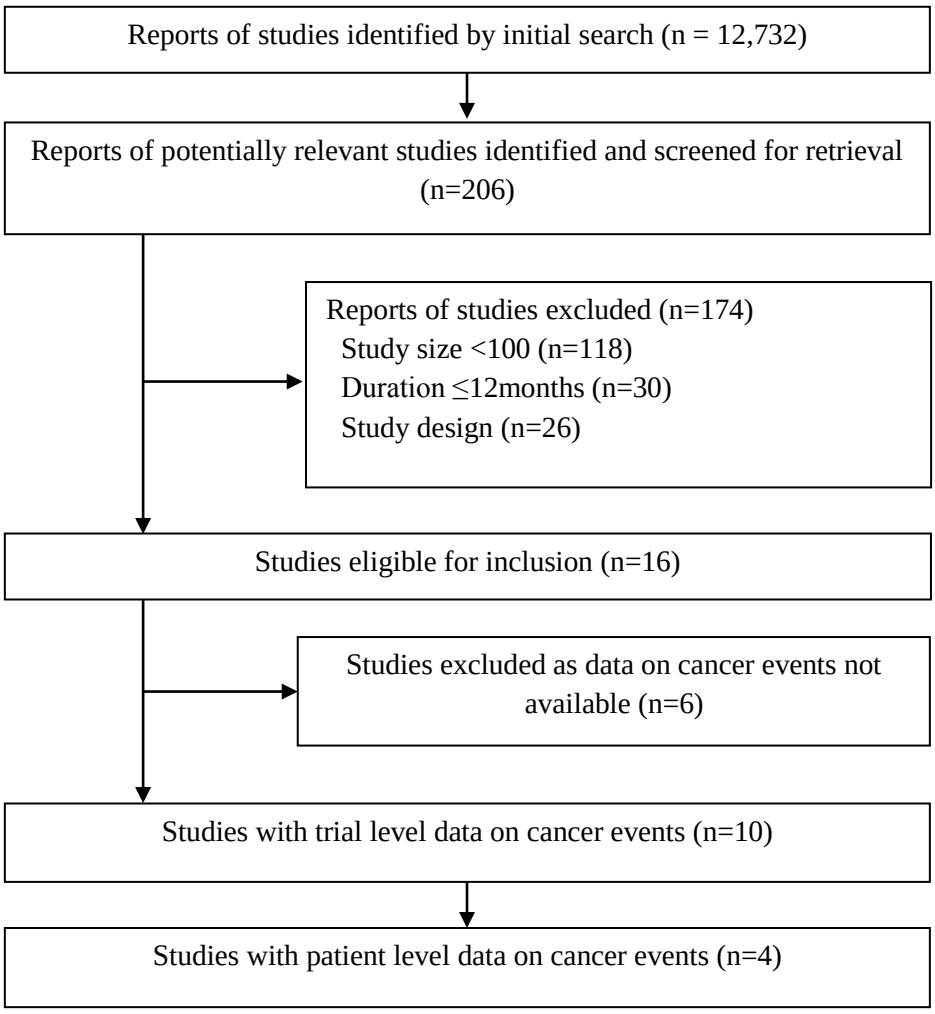

Figure 7.1 PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow chart of studies. The initial search was in November 2007 with 9,358 reports identified, 173 reports of potentially relevant studies retrieved, 150 reports excluded and twenty-three reports of fifteen individual studies identified. The search was updated in March 2010 and February 2012: a further 3,374 reports were identified, thirty-three reports retrieved and one new study identified.

Table 7.1 Characteristics of 16 studies eligible for inclusion in meta-analysis.

Studies	No in calcium group / No in control group	Daily dose and supplement type	Trial duration (years)	Primary endpoint	Baseline mean age (years)	% female
Patient level data on cancer outcomes:						
Reid 1993[14]	68/67	1g lactogluconate-carbonate	4	Bone mineral density	58	100
Grant 2005 [270, 333]	2617/2675	1g carbonate	4*	Low trauma fracture	77	85
Reid 2006 [137, 336]	732/739	1g citrate	5	Clinical fracture	74	100
Reid 2008 [62]	216/107	0.6g or 1.2g citrate	2	Spine bone mineral density	56	0
Subtotal/average†	3633/3588	-	4.1	-	75.5	87.1
Trial level data on cancer outcomes ‡:						
Riggs 1998 [294]	119/117	1.6g citrate	4	Bone mineral density	66	100
Baron 1999 [267, 269]	464/466	1.2g carbonate	4	Colorectal adenoma	61	28
Bonithon-Kopp 2000 [268]	204/212	2g lacto-gluconate carbonate	3	Colorectal adenoma	59	37
Lappe 2007 [266]	445/288	1.4g citrate or 1.5g carbonate	4	Fracture incidence	67	100
Bonnick 2007 [337]	282/281	1.25g carbonate	2	Spine bone mineral density	66	100
Chailurkit 2010 [136]	201/196	0.5g carbonate	2	PTH and bone mass	66	100
Subtotal/average†	1715/1560	-	3.3	-	63.7	67.8
Total/average†	5348/5148	-	3.9	-	72.3	82.0
No data on cancer outcomes:						
Smith 1989 [339]	84/85	1.5g carbonate	4	Arm bone mineral density	51	100
Dawson-Hughes 1990 [13]	238/123	0.5g carbonate or citrate	2	Spine bone mineral density	58	100
Elders 1991 [340]	198/97	1g or 1.2g lactogluconate-carbonate or citrate	2	Spine bone mineral density	NA	100
Recker 1996 [16]	95/102	1.2g carbonate	4	Vertebral fracture	74	100
Peacock 2000 [338]	126/135	0.75g citrate	4	Hip bone mineral density	76	72
Prince 2006 [332]	730/730	1.2g carbonate	5	Osteoporotic fracture	75	100
Subtotal/average†	1471/1272	-	4.1	-	68.5	97.4

NA, not available. * Mean duration was 45 months, with all participants followed for at least 2 years. †Weighted by person years of follow-up. ‡Complete trial-level data on total cancer events was available for seven studies [14, 62, 137, 266, 267, 333, 337] and partially complete trial-level data was available for three studies [136, 268, 294]

Table 7.2 Baseline variables in trials with patient or trial level data available for cancer outcomes.

Studies	Dietary calcium (mg/day)	25-hydroxyvitamin D (nmol/L)	Weight (kg)	BMI (kg/m^2)	Current smoker (%)	History of colorectal adenoma (%)
Reid 1993[14]	750 (290)	93 (37)	65 (9)	25 (3)	10	NA
Riggs 1998 [294]	710 (290)	80 (25)	NA	NA	NA	NA
Baron 1999 [269]	880 (440)	73 (27)	82 (15)	27 (4)	19	100
Bonithon-Kopp 2000 [268]	980 (380)	NA	NA	NA	NA	100
Grant 2005 [333] †	820 (350)	38 (16)	65 (12)	NA	12	NA
Reid 2006 [137]	860 (390)	54 (18)	67 (11)	26 (4)	3	NA
Reid 2008 [62]	870 (450)	92 (33)	83 (12)	26 (3)	3	NA
Lappe 2007 [266]	1070*	72 (20)	77 (15)	29 (6)	NA	NA
Bonnick 2007 [337]	1240 (580)	NA	NA	NA	0.4	NA
Chailurkit 2010 [136]	375 (210)	69 (19)	59 (8)	25 (3)	NA	NA

NA, not available. Values are mean (standard deviation) unless stated otherwise. * Value is median. † 25-hydroxyvitamin D measured in a sample of 60 participants

Trial-level analysis

Seven trials provided complete trial-level data on total cancer events and were included in the primary analysis. The number of people with cancer events in each study by treatment allocation is shown in Table 7.3 and the results of the trial-level analysis are shown in Figures 7.2 - 7.6. Allocation to calcium supplements had no effect on the risk of total cancer, colorectal cancer, breast cancer or cancer-related mortality. Allocation to calcium supplements was associated with a significant decrease in the risk of prostate cancer. A further three trials [136, 268, 294] for total cancer, and two trials [136, 294] for colorectal and breast cancer had data only for subgroups of participants and were included in a sensitivity analysis that included data from all ten trials. Including data from these further trials did not change the results for any endpoint. Publication bias was not evident on inspection of funnel plots in any analysis.

Table 7.3 Number of people with cancer and cancer-related mortality by treatment group

Studies	Calcium group						Control group					
	N randomised	Total cancer*	Colorectal cancer	Breast cancer	Prostate cancer	Cancer mortality	N randomised	Total cancer*	Colorectal cancer	Breast cancer	Prostate cancer	Cancer mortality
Reid 1993[14]	68	4	1	0	NP	0	67	2	1	1	NP	0
Riggs 1998 [294] †	119	3	0	0	NP	NA	117	2	0	1	NP	NA
Baron 1999 [267, 269] ‡	464	20	3	0	9	7	466	30	5	4	15	9
Bonithon-Kopp 2000 [268] §	204	4	0	NA	NA	4	212	4	1	NA	NA	4
Grant 2005 [270, 333] ‖	1311	94	15	14	4	50	1332	84	7	11	8	51
Grant 2005 VitD [270, 333] ¶	1306	83	20	13	4	41	1343	92	13	14	7	45
Reid 2006 [137, 336]	732	46	7	14	NP	7	739	42	6	9	NP	7
Lappe 2007 [266]	445	17	0	6	NP	NA	288	20	2	8	NP	NA
Bonnick 2007 [337]	282	6	2	2	NP	NA	281	3	0	0	NP	NA
Reid 2008 [62]	216	0	0	NP	0	0	107	1	0	NP	1	0
Chailurkit 2010 [136]†	201	0	0	0	NP	NA	196	2	0	1	NP	NA
Total	5348	277	48	49	17	109	5148	282	35	49	31	116

NA, not available. NP, not applicable for breast cancer as all subjects male, or for prostate cancer as all subjects female. * Total cancer excluding non-melanoma skin cancers. † Unpublished trial-level data on reasons for study withdrawals provided by author. ‡ Unpublished trial-level data on total cancers, breast cancers and cancer-related mortality provided by the author, data on colorectal cancers and prostate cancers from published data. § Unpublished trial-level data on cancer deaths provided by the author, data on colorectal cancers from published data. ‖ Calcium versus placebo arms in Randomised Evaluation of Calcium or Vitamin D (RECORD) study. ¶ Calcium plus vitamin D and placebo plus vitamin D arms in Randomised Evaluation of Calcium or Vitamin D (RECORD) study.

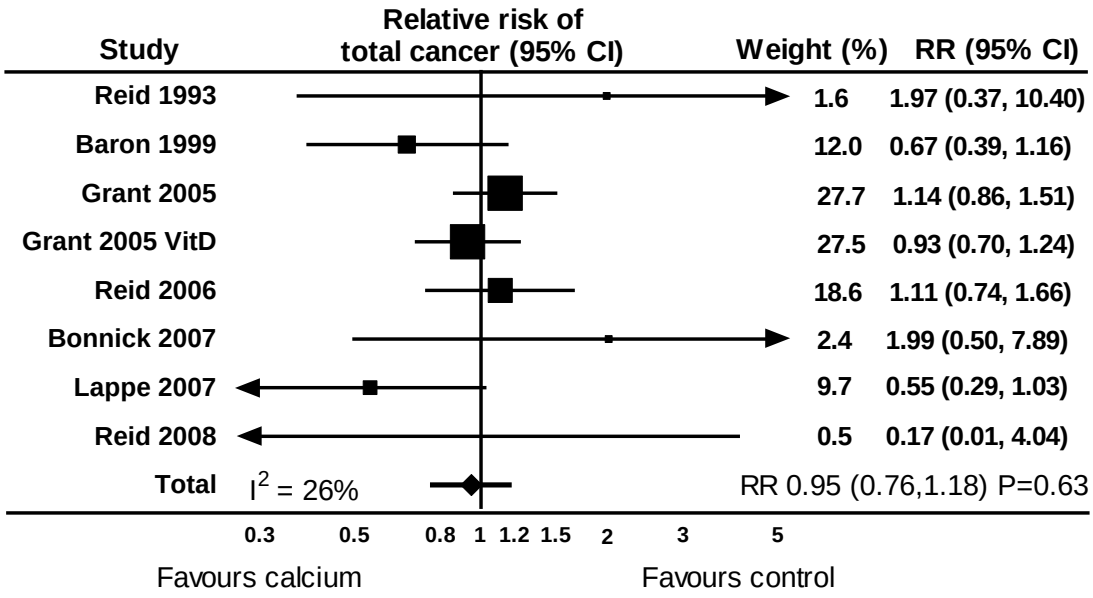

Figure 7.2 Random-effects model of calcium supplementation on total cancer events. Grant 2005 is the calcium versus placebo arms of this study, and Grant 2005 vitamin D (VitD) is the calcium plus VitD versus VitD-only arms [333]. RR, relative risk.

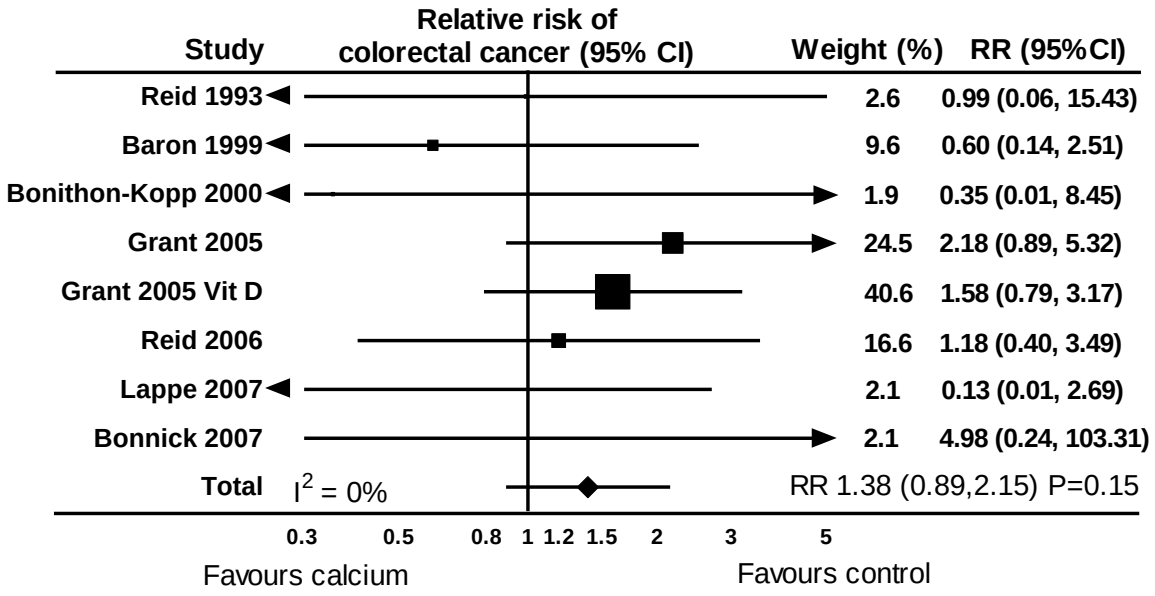

Figure 7.3 Random-effects model of calcium supplementation on colorectal cancer events. Full trial-level data were available for eight studies for colorectal cancer. However, one study is not shown as no colorectal cancer events occurred [62]. Grant 2005 is the calcium versus placebo arms of this study, and Grant 2005 vitamin D (VitD) is the calcium plus VitD versus VitD-only arms [333]. RR, relative risk.

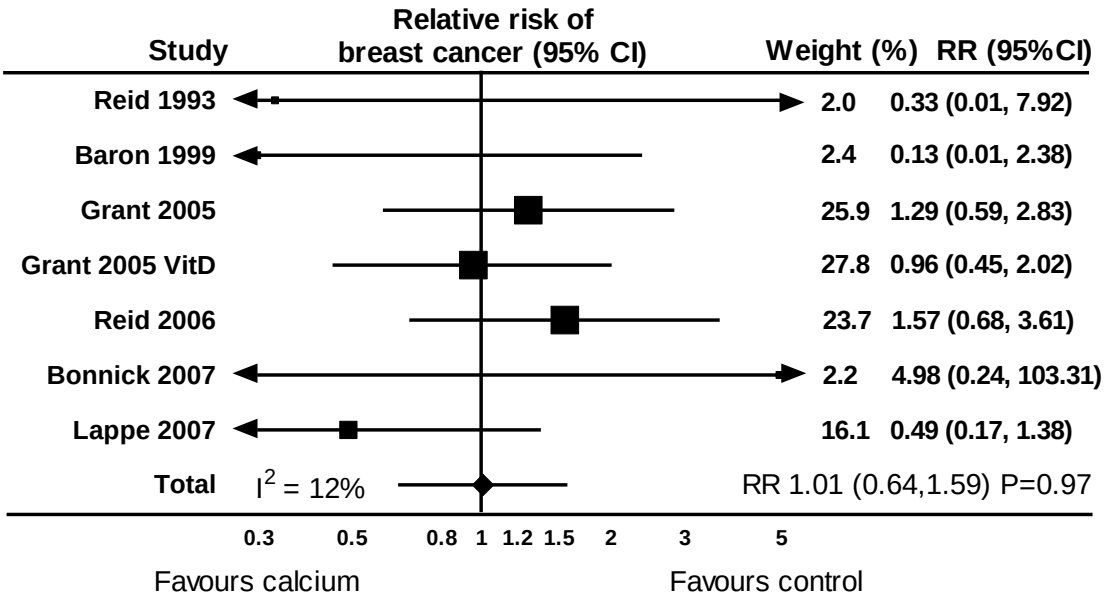

Figure 7.4 Random-effects model of calcium supplementation on breast cancer events. Grant 2005 is the calcium versus placebo arms of this study, and Grant 2005 vitamin D (VitD) is the calcium plus VitD versus VitD-only arms. RR, relative risk.

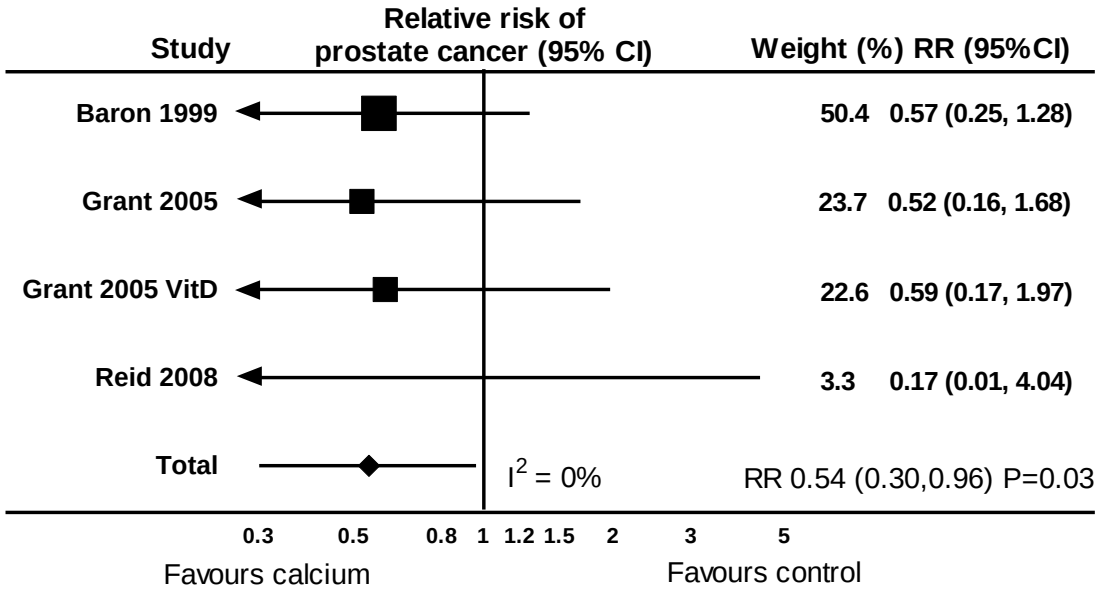

Figure 7.5 Random-effects model of calcium supplementation on prostate cancer events. Grant 2005 is the calcium versus placebo arms of this study, and Grant 2005 vitamin D (VitD) is the calcium plus VitD versus VitD-only arms. RR, relative risk.

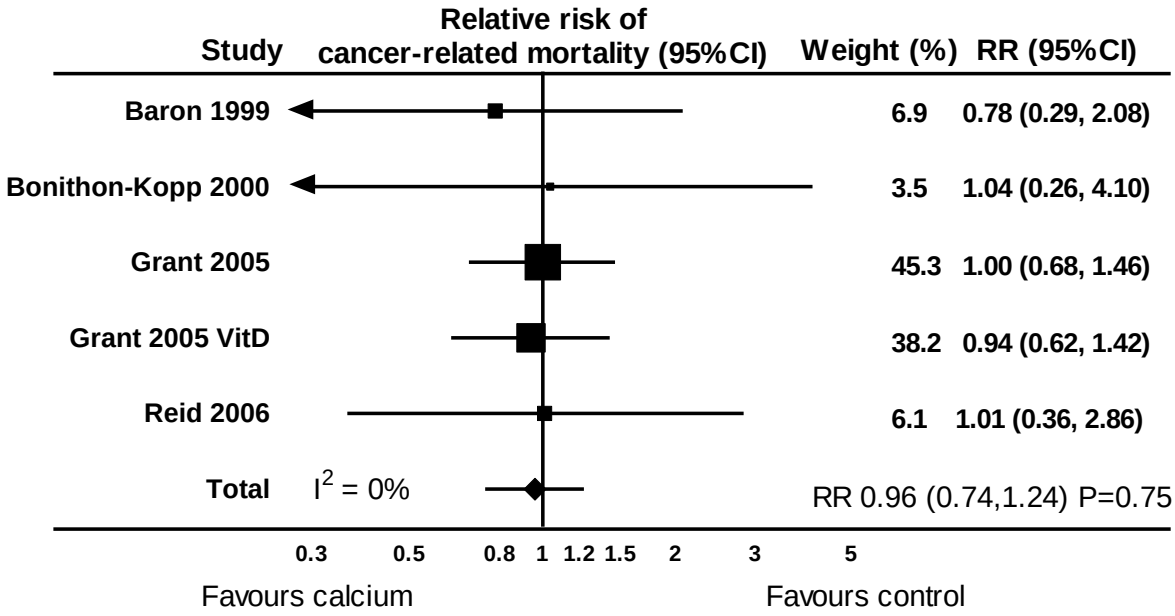

Figure 7.6 Random-effects models of calcium supplementation on cancer mortality. Full trial-level data were available for six studies for cancer mortality. However, two studies are not shown as no cancer-related mortality occurred [14, 62]. Grant 2005 is the calcium versus placebo arms of this study, and Grant 2005 vitamin D (VitD) is the calcium plus VitD versus VitD-only arms. RR, relative risk.

Patient-level analysis

Four trials provided patient-level data and were included in a secondary patient-level analysis. Selected baseline characteristics are shown in Table 7.4, and the results in Table 7.5 and Figure 7.7. The median (interquartile range) duration of follow-up in both groups was 3.5 years (2.6 to 4.4). Allocation to calcium supplements had no effect on the risk of total cancer, breast cancer or cancer-related mortality. Allocation to calcium supplements significantly increased the risk of colorectal cancer, and nonsignificantly decreased the risk of prostate cancer. Adjusting for prespecified covariates likely to be related to cancer outcomes, with data available for more than 80% of participants, (age, sex, smoking status and weight) did not change these results, nor did including trials in which data were obtained only from verified sources. Repeating the models including a latent period of 1 or 2 years, or censoring the first 50% of cancer events, to attempt to adjust for cancers that were present but undetected at baseline, moved the results for colorectal cancer and prostate cancer towards significance but did not alter the results in any other way (Table 7.5). In prespecified subgroup analyses, no interactions were identified between treatment allocation and age, sex, vitamin D status, smoking status, dietary calcium intake and supplement type for any cancer endpoint.

Table 7.4 Baseline characteristics of participants in four studies included in the patient level analysis by treatment allocation.

Characteristics	**Calcium group** **N = 3633**	**Placebo group** **N = 3588**
Median (interquartile range) age (years)	75.2 (72-80)	75.6 (72-80)
Women (%)*	83.0	85.6
White ethnicity (%)	99.1	99.4
Mean (SD)weight (kg)	66.5 (12.9)	66.2 (12.7)
Mean (SD) dietary calcium (mg/day)	830 (366)	827 (362)
Mean (SD) 25-hydroxyvitamin D (nmol/L)*	63.6 (28.8)	60.7 (28.0)
Current smoker (%)	9.9	9.1

*Proportion of women was significantly higher in placebo group because one study that only involved men had a 2:1 ratio of allocation to calcium or placebo. No other differences existed between groups.
† Data available from four studies for 1050 participants in the calcium groups and 952 participants in the placebo groups.

Table 7.5 Results of patient level analysis. Number of people with cancer events.

	Calcium N = 3633	**Placebo N = 3588**	**Hazard Ratio (95% CI)**	***P* value**
Total events				
Total cancer	227	221	1.07 (0.89, 1.28)	0.50
Colorectal cancer	43	27	1.63 (1.01, 2.64)	0.046*
Breast cancer	41	35	1.27 (0.81, 2.02)	0.30
Prostate cancer	8	16	0.49 (0.21, 1.14)	0.098
Cancer related mortality	98	103	0.98 (0.74, 1.29)	0.86
1 year latent period †				
Total cancer	172	160	1.12 (0.90, 1.39)	0.30
Colorectal cancer	34	19	1.84 (1.05, 3.22)	0.03*
Breast cancer	33	25	1.47 (0.86, 2.51)	0.15
Prostate cancer	4	11	0.34 (0.11, 1.08)	0.07
Cancer related mortality	77	75	1.05 (0.77, 1.45)	0.75
2 year latent period ‡				
Total cancer	112	96	1.24 (0.94, 1.63)	0.13
Colorectal cancer	19	14	1.39 (0.70, 2.77)	0.35
Breast cancer	22	17	1.51 (0.78, 2.90)	0.22
Prostate cancer	3	4	0.74 (0.17, 3.31)	0.69
Cancer related mortality	49	42	1.2 (0.79, 1.81)	0.39
First 50% of events censored				
Total cancer	120	104	1.20 (0.94, 1.59)	0.14
Colorectal cancer	20	15	1.37 (0.70, 2.67)	0.36
Breast cancer	22	16	1.61 (0.83, 3.15)	0.16
Prostate cancer	3	9	0.31 (0.08, 1.14)	0.08
Cancer related mortality	50	51	1.01 (0.68, 1.49)	0.98

*P <0.05

† Events during first year censored

‡ Events during first and second year censored

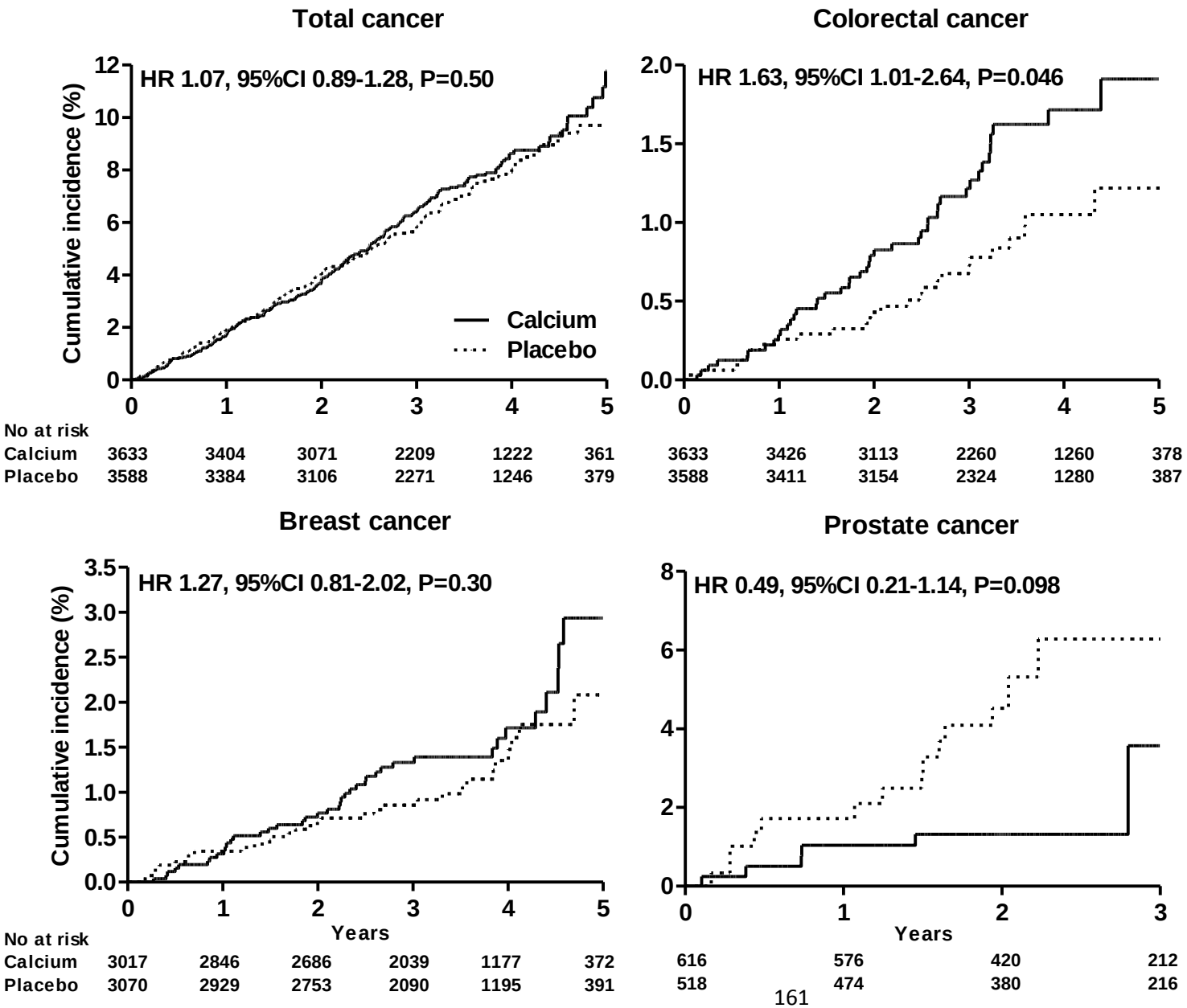

Figure 7.7 Cumulative incidence of (a) total cancer, (b) colorectal cancer, (c) breast cancer and (d) prostate cancer in four studies that contributed patient-level data.

7.4 DISCUSSION

In this meta-analysis of 10,500 participants from ten trials, calcium supplements without coadministered vitamin D did not alter the risk of total cancer, breast cancer or cancer-related mortality over 4 years. Calcium supplements significantly reduced prostate cancer risk; however this was based on small numbers of events. Calcium supplements did not alter colorectal cancer risk in the trial-level analysis; while there was an increased risk in the patient-level analysis, this was based on small numbers of events. Including a latent period of 1 or 2 years, and censoring the first 50% of events did not meaningfully alter the results.

The association between dietary calcium intake and cancer risk has been the subject of numerous observational studies, however few RCTs of calcium supplements have examined cancer incidence as an outcome and none as a primary endpoint. In a 4 year trial of 1,179 postmenopausal women, Lappe et al [266] reported a non-significant 47% reduction in total cancer risk with calcium monotherapy, and a significant 60% reduction with calcium with vitamin D. There was no reduction in cancer risk in the calcium with vitamin D group compared with the calcium monotherapy group ($p = 0.46$), suggesting that calcium supplements were responsible for the protective effects. I was unable to confirm reductions in cancer risk with calcium monotherapy over an equivalent 4 year period in this meta-analysis, with consistent findings in both trial- and patient-level analyses. Compared with the trial by Lappe et al, the population in this meta-analysis was older (72 versus 67 years), but had a lower annualised incidence of cancer in the control group (1.4% versus 1.7%). This might suggest that the positive findings in the Lappe trial resulted from an unexplained high rate of cancer in their control group [341]. Alternatively, the reduction in cancer risk in their trial may have been a chance finding, due to the small number of cancer events ($n = 50$, compared with 550 in the present analysis). Consistent with the findings of this meta-analysis, a recent analysis of RECORD [270], a trial of 5292 older people randomised to calcium, vitamin D, calcium with vitamin D, or placebo, found no effect of calcium (with or without vitamin D) on the risk of total cancer incidence or cancer-related mortality.

A protective effect of calcium with vitamin D supplements against total, breast and possibly colorectal cancer was suggested in a recent re-analysis of the WHI data by Bolland et al [30]. As calcium and vitamin D were administered together in that trial it was not possible to determine which agent was responsible for the protective effects. The results of this meta-

analysis suggest calcium supplements for 4 years without coadministered vitamin D have no effect on the risk of total cancer, which might indicate vitamin D, or a combination of both agents, was responsible for the observed reduction in risk in the WHI re-analysis. However, it is also possible that this meta-analysis was underpowered and/or of too short duration to detect a small effect of calcium on total cancer risk. The total cancer endpoint in the WHI re-analysis was based on 1,300 cancer events over 7 years among 15,600 women, whereas the present analysis included 550 cancer events over 4 years among 10,500 men and women. The lack of an effect of calcium on total cancer in this meta-analysis suggests that if calcium does have an effect on total cancer risk, it is small.

In contrast with earlier observational studies [252-254] and colorectal adenoma chemoprevention trials [267, 342], I found no evidence that calcium monotherapy reduces colorectal cancer risk. This may have been due to the duration of the trials. If colorectal cancer has a latency period of 10 - 20 years, participants diagnosed during each trial may have had early stage cancer that was present but asymptomatic and undetected at baseline. While calcium may inhibit colorectal cancer initiation, such as adenoma formation [267], little research has examined its effects on cancer progression. In polyp-bearing participants, calcium and antioxidants had no effect on existing adenoma growth, but significantly reduced new adenoma development over 3 years [342]. The present meta-analysis included two trials that had participants with a history of colorectal adenoma. Because of the small number of colorectal cancer events in these trials (three in the combined calcium groups and six in the combined control groups), I was unable to investigate whether the effect of calcium on colorectal cancer was different in these participants.

There was some evidence of an increased colorectal cancer risk with calcium in the present analysis, although this was only statistically significant in the patient-level analysis. The increased risk may have resulted from increased screening for colorectal cancer in the group allocated to calcium, as change in bowel habits is an early symptom of colorectal cancer [343], and calcium supplements cause gastrointestinal side-effects [344]. While the increased risk could reflect an effect of calcium on colorectal cancer progression, the small number of colorectal cancer events and lack of statistical significance in the primary analysis suggest it might be a chance finding.

There was a reduction in prostate cancer risk with calcium, which was largely based on two trials [269, 333]. In a more detailed analysis of one of these trials [269], calcium had no effect on prostate cancer risk over 10 years (4 years of treatment, 6 years of post-treatment follow-up), but reduced risk by 48% during the first 6 years. These results suggest that the positive association between calcium intake and increased prostate cancer risk suggested by some [257-259] but not all [263, 264] observational studies may be unrelated to calcium, and instead due to other factors correlated with calcium intake, such dairy product intake. Men with higher dietary calcium intakes might also receive more screening for prostate cancer. The small number of prostate cancer events means these findings should be interpreted cautiously. If calcium does protect against prostate cancer, this could be mediated through the calcium-sensing receptor, found on prostate cells [345], or indirectly through PTH, which has been implicated in prostate carcinogenesis [346].

This meta-analysis has some limitations. Cancer was not a primary outcome of any included study, and cancer events were not collected in a standardized manner. However, unless there was differential misclassification or misreporting of cancer events in people allocated to calcium (as I suggested for colorectal cancer) this should not have affected these results. No data for cancer events were available for six trials comprising 20% of participants. With one exception, these trials were small, and the consistency of the findings suggests the addition of these trials would not have affected these results.

In summary, I found no evidence that calcium supplementation without coadministered vitamin D influences total cancer risk over 4 years. The differences between the findings of this meta-analysis and those of a recent re-analysis of the WHI suggesting benefits of coadministered calcium and vitamin D on cancer incidence, might be attributable to the coadministration of vitamin D in that trial. However, I cannot rule out that this meta-analysis was underpowered or that the duration of trials was too short to detect a small effect of calcium supplements. Any future trials of calcium supplements on cancer incidence would need to be very large and of a long duration to detect clinically relevant effects of calcium supplements on cancer risk.

CHAPTER 8: CONCLUSIONS

Calcium supplements have been widely used for bone health. However, recent evidence suggests calcium supplements increase the risk of myocardial infarction and stroke [29]. The mechanism underlying the increase in risk is presently unclear, but could involve the elevation in serum calcium that follows the ingestion of a calcium supplement. Increased serum calcium has been positively associated with cardiovascular risk in observational studies [179, 180]. An acute elevation in serum calcium is known to occur following the ingestion of a bolus of supplemental calcium, however the duration of this elevation and whether it is diminished over time is unknown. In Chapter 3, I demonstrated that 1000 mg of supplemental calcium elevated serum calcium for at least 8 hours, and that this elevation was not diminished after 3 months of continuous use. These findings indicate that calcium supplement users will have persistent elevations in serum calcium for the duration of their use of calcium supplements, and that those taking doses of 1000 mg will have elevated serum calcium for at least 8 hours each day.

A limitation of this trial and previous acute trials was that a 1000 mg dose of calcium was studied, to maximise differences between the interventions. Calcium supplements are, however, commonly taken in smaller divided doses, and may have a smaller calcaemic effect than that usually reported. It has also been suggested that calcium supplements should be taken with meals, in order to blunt their calcaemic effect [313]. In Chapter 5, I demonstrated that 500 mg of calcium from a supplement elevated serum calcium for at least 6 hours, and that this elevation was delayed, but not diminished, when a supplement was taken with a meal. These findings suggest that individuals taking two doses of 500 mg of calcium each day will have some elevation in serum calcium for at least 12 hours. These findings do not provide support for recommendations to take calcium supplements with meals.

In addition to calcium supplements, fortified foods, such as fruit juice, may be used to supplement calcium intake. In Chapter 5, I found that the elevation in serum calcium was comparable after 500 mg of calcium from a supplement and fortified juice. It is therefore possible that calcium-fortified foods might be associated with the same cardiovascular risks as supplements; however they might be used in smaller amounts. In contrast to calcium supplements, most observational studies have not found a relationship between dietary calcium intake and increased cardiovascular risk [36, 170, 173-175, 177]. These differences

might be due to a smaller calcaemic effect of calcium from dietary sources, although this has not been well-studied. In Chapter 5 I found that the elevation in serum calcium following 500 mg of calcium from dairy products was smaller than calcium from a supplement. Furthermore, as the dose administered would represent a large amount of calcium from a single meal, the acute changes in serum calcium associated with high calcium diets are likely to be even smaller.

Calcium has a diverse range of biological functions. As such, there exist a number of ways in which the large and sustained increase in calcium intake that occurs with calcium supplementation, or the associated elevations in serum calcium, could influence cardiovascular health. Few studies have examined changes in indices of cardiovascular risk following the ingestion of a calcium supplement. In Chapter 4, I reported a trend for blood pressure to be acutely higher following a calcium supplement compared with a control. This contrasts with the findings of long-term trials, in which modest reductions in blood pressure have been observed with calcium supplementation [153, 159]. However, it is consistent with the acute hypertensive effect of calcium infusion [194, 195]. I also found evidence that the coagulability of blood was increased after the ingestion of a calcium supplement. These novel findings could explain the increase in cardiovascular risk associated with calcium supplementation; however the small numbers of participants and their borderline significance means they require confirmation in larger studies.

The suggested adverse effects of calcium supplements on cardiovascular and kidney stone risk have led to recommendations that, for bone health, calcium should be obtained through the diet [31, 32, 149]. However, dietary intakes of calcium often fall below the recommended levels [22, 23, 347] and high dairy diets may not be acceptable to many people. In Chapter 6 I found that dietary calcium intake was not associated with bone loss or medium-term fracture risk fracture in postmenopausal women. There was a weak association between dietary calcium intake and absolute BMD, the clinical significance of which is unclear. These findings are consistent with most previous prospective cohort studies, suggesting that dietary calcium intake will not influence fracture risk in older adults. These observations support the continued omission of dietary calcium intake from fracture risk calculators in clinical use. However, the importance of increasing dietary calcium intake for bone health is widely promoted in the lay and scientific literature [65-67]. It is important that recommendations to improve bone health are evidence based, and my findings and those of most previous studies

suggest there is currently no convincing evidence that dietary calcium intake in older adults influences bone health.

The modest but beneficial effects of calcium supplementation on bone loss and fracture risk are often used as evidence that higher dietary calcium intakes will benefit bone health. This view presupposes that intakes of dietary calcium are deficient, and that the effects of calcium supplements are due to the correction of this deficiency. If true, it should follow that the effects of calcium supplements on bone are greater in individuals with lower dietary calcium intakes, and are smaller or non-existent in those with higher intakes. However, in Chapter 6 I found that the reduction in bone loss associated with calcium supplementation was comparable across the range of dietary calcium intakes studied, including participants who would have been classified as markedly calcium deficient (mean intake 410 mg/day) and calcium replete (1460 mg/day). The lack of an influence of background dietary calcium intake is a consistent finding in most large calcium supplementation trials [331-333, 348]. These findings suggest the effects of calcium supplements on bone are related to calcium supplementation itself, rather than the correction of a nutritional inadequacy.

In Chapter 2 I suggested several reasons for the apparent differences between the effects of calcium supplementation and dietary calcium on bone health (and cardiovascular health). Firstly, calcium supplements may elevate serum calcium more than dietary calcium. Consistent with this, in Chapter 5, I reported that a dairy meal elevated serum calcium less than an equivalent dose of calcium from a supplement or fortified juice. The smaller elevation in serum calcium might translate into a smaller reduction in PTH and bone turnover over time. However, in Chapter 3, I found that MCH also resulted in a smaller elevation in ionised calcium than conventional calcium supplements, but suppressed bone turnover comparably. Thus, even very small increases in serum calcium might be sufficient to lower bone turnover. Calcium supplementation also represents a dramatic increase in calcium intake by 1000 mg/day, while dietary calcium intakes may be more stable. It could be the change in calcium intake which is important, as the effects of calcium supplements are greatest in the 1-3 years after supplementation is initiated [137]. Finally, calcium supplementation results in the addition of calcium in isolation to the diet, while calcium from the diet will be supplied with other nutrients, which may influence calcium metabolism. For these reasons, the effects on bone of an intervention with a large concentrated bolus of calcium might be better viewed as pharmacological rather than physiological. The effects of calcium supplements on bone loss in clinical trials are very similar to that of a weak anti-resorptive pharmaceutical.

Finally, dietary calcium has been widely studied in relation to cancer risk; however, few trials of calcium supplements have examined cancer outcomes. In one influential trial, calcium supplementation halved cancer risk over 4 years of treatment; however the number of cancer events was very small [266]. In a recent re-analysis of the WHI, calcium with vitamin D supplementation was associated with small reductions in the risk of total and breast cancer, and possibly colorectal cancer [30]. Such reductions in cancer risk could be clinically important; however it was unclear whether the protective effects were attributable to calcium or vitamin D. In a meta-analysis of RCTs in Chapter 7, I found no effect of calcium monotherapy on the risk of total or breast cancer over 4 years. There was a trend for an increased risk of colorectal cancer, and a decreased risk of prostate cancer, however these endpoints were based on small numbers of participants. These findings could indicate that vitamin D was responsible for the reduction in cancer risk in the WHI.

There are a number of options for further research in these areas. The trend for higher blood pressure and increased blood coagulability acutely following a calcium supplement require further investigation. I plan to re-examine these outcomes in a cross-over trial of postmenopausal women. Knowledge of the relationship between dietary calcium intake and bone health would be improved by further studies in this area. I plan to carry out analyses of two further databases from RCTs to examine relationships between dietary calcium intake, PTH, BMD and fracture risk. While the findings of this thesis suggest that current dietary calcium intakes do not influence bone loss in older women, it remains possible that dietary calcium may have a relationship with bone mass during growth. This could explain the small association I found between dietary calcium intake and BMD, but studies in this area have so far been inconsistent [349, 350]. It is also unclear how low dietary calcium intake can be before bone health is compromised. As obligatory losses of calcium cannot be reduced to zero, there must be a threshold at which calcium balance becomes negative and bone loss ensues. This may explain the increased risk of fracture in the lowest category of dietary calcium intake in some observational studies [77]. This is an important question as some elderly may have very low calcium intakes [64]. Finally, the possible protective effect of vitamin D supplementation on cancer risk will be explored further by others in large trials of vitamin D that are currently ongoing.

The findings described in this thesis of the acute and sustained elevation in serum calcium following a calcium supplement, the different calcaemic effects of calcium from food and calcium from supplements and fortified juice, and the adverse trends in blood pressure and

blood coagulation following a calcium supplement, all indicate that the safest way for individuals to obtain calcium is through the diet. However, the findings of this thesis also indicate that dietary calcium intake does not influence bone loss or fracture risk in older women, and the amount of calcium required may therefore be lower than that currently recommended. Collectively, these findings suggest that for bone and cardiovascular health, individuals can consume their normal diet. Most diets will provide adequate calcium, in a form that is safe.

BIBLIOGRAPHY

1. Albright, F., P.H. Smith, and A.M. Richardson, *Postmenopausal osteoporosis - Its clinical features.* Journal of the American Medical Association, 1941. **116**: p. 2465-2474.
2. Nicholls, L. and A. Nimalasuriya, *Adaptation to a low calcium intake in reference to the calcium requirements of a tropical population.* Journal of Nutrition, 1939. **18**(6): p. 563-577.
3. Walker, A.R.P., *Some Aspects of Nutritional Research in South-Africa.* Nutrition Reviews, 1956. **14**(11): p. 321-324.
4. Pathak, C.L., *Nutritional Adaptation to Low Dietary Intakes of Calories, Proteins, Vitamins, and Minerals in the Tropics.* American Journal of Clinical Nutrition, 1958. **6**(2): p. 151-158.
5. Hegsted, D.M., I. Moscoso, and C. Collazos, *A study of the minimum calcium requirements of adult men.* J Nutr, 1952. **46**(2): p. 181-201.
6. *Recommended dietary allowances, 1953.* Nutr Rev, 1954. **12**(8): p. 240-2.
7. National Academy of Sciences, *Recommended dietary allowances. Revised 1953.* , National Research Council 1953: Washington D.C.
8. World Health Organisation and Food and Agriculture Organisation of the United Nations, *Calcium requirements: report of an FAO/WHO Expert Group, Rome, Italy, 23 to 30 May 1961*, World Health Organisation 1962: Geneva.
9. Food and Agriculture Organisation of the United Nations and World Health Organisation, *Handbook on human nutritional requirements*, World Health Organisation 1974: Geneva.
10. Heaney, R.P., R.R. Recker, and P.D. Saville, *Menopausal changes in calcium balance performance.* J Lab Clin Med, 1978. **92**(6): p. 953-63.
11. Heaney, R.P., R.R. Recker, and P.D. Saville, *Calcium balance and calcium requirements in middle-aged women.* Am J Clin Nutr, 1977. **30**(10): p. 1603-11.
12. Chapuy, M.C., et al., *Vitamin D3 and calcium to prevent hip fractures in the elderly women.* N Engl J Med, 1992. **327**(23): p. 1637-42.
13. Dawson-Hughes, B., et al., *A controlled trial of the effect of calcium supplementation on bone density in postmenopausal women.* N Engl J Med, 1990. **323**(13): p. 878-83.
14. Reid, I.R., et al., *Effect of calcium supplementation on bone loss in postmenopausal women.* N Engl J Med, 1993. **328**(7): p. 460-4.
15. Chevalley, T., et al., *Effects of calcium supplements on femoral bone mineral density and vertebral fracture rate in vitamin-D-replete elderly patients.* Osteoporos Int, 1994. **4**(5): p. 245-52.
16. Recker, R.R., et al., *Correcting calcium nutritional deficiency prevents spine fractures in elderly women.* J Bone Miner Res, 1996. **11**(12): p. 1961-6.
17. Food and Nutrition Board and Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, *Dietary Reference Intakes for Calcium, Phosphorous, Magnesium, Vitamin D and Fluoride.*, National Academy Press 1997: Washington DC.
18. Food and Agriculture Organisation of the United Nations and World Health Organisation, *Human vitamin and mineral requirements. Report of a joint FAO/WHO expert consultation, Bangkok, Thailand.*, World Health Organisation 2002: Rome.
19. National Health and Medical Research Council and Ministry of Health, *Nutrient Reference Values for Australia and New Zealand.*, Department of Health and Aging 2006: Canberra.

20. Committee on Medical Aspects of Food and Nutrition Policy and Department of Health, *Dietary reference values for food, energy and nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food.*, HMSO 1991: London.
21. Committee on Medical Aspects of Food and Nutrition Policy, *Report on Health and Social Subjects 49: Report of the Subgroup on Bone Health, Working Group on the Nutrition Status of the Population of the Committee on Medical Aspects of Food and Nutrition Policy.*, Department of Health 1998: London.
22. University of Otago and Ministry of Health, *A Focus on Nutrition: Key findings of the 2008/09 New Zealand Adult Nutrition Survey.*, Ministry of Health 2011: Wellington.
23. Australian Instutite of Health and Welfare, *Australia's food and nutrition 2012.*, AIHW, 2012: Canberra.
24. Bailey, R.L., et al., *Dietary supplement use is associated with higher intakes of minerals from food sources.* Am J Clin Nutr, 2011. **94**(5): p. 1376-81.
25. Brownie, S., *Predictors of dietary and health supplement use in older Australians.* Aust J Adv Nurs, 2006. **23**(3): p. 26-32.
26. Berner, L.A., F.M. Clydesdale, and J.S. Douglass, *Fortification contributed greatly to vitamin and mineral intakes in the United States, 1989-1991.* J Nutr, 2001. **131**(8): p. 2177-83.
27. Forshee, R.A., P.A. Anderson, and M.L. Storey, *Changes in calcium intake and association with beverage consumption and demographics: comparing data from CSFII 1994-1996, 1998 and NHANES 1999-2002.* J Am Coll Nutr, 2006. **25**(2): p. 108-16.
28. Institute of Medicine, *Dietary Reference Intakes for Calcium and Vitamin D*. 2011, The National Academies Press: Washington, DC.
29. Bolland, M.J., et al., *Calcium supplements with or without vitamin D and risk of cardiovascular events: reanalysis of the Women's Health Initiative limited access dataset and meta-analysis.* BMJ, 2011. **342**: p. d2040.
30. Bolland, M.J., et al., *Calcium and vitamin D supplements and health outcomes: a reanalysis of the Women's Health Initiative (WHI) limited-access data set.* Am J Clin Nutr, 2011. **94**(4): p. 1144-9.
31. Manson, J.E. and S.S. Bassuk, *Calcium supplements: do they help or harm?* Menopause, 2014. **21**(1): p. 106-8.
32. Bauer, D.C., *Clinical practice. Calcium supplements and fracture prevention.* N Engl J Med, 2013. **369**(16): p. 1537-43.
33. Curhan, G.C., et al., *Comparison of dietary calcium with supplemental calcium and other nutrients as factors affecting the risk for kidney stones in women.* Ann Intern Med, 1997. **126**(7): p. 497-504.
34. Curhan, G.C., et al., *A prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones.* N Engl J Med, 1993. **328**(12): p. 833-8.
35. Wallace, R.B., et al., *Urinary tract stone occurrence in the Women's Health Initiative (WHI) randomized clinical trial of calcium and vitamin D supplements.* Am J Clin Nutr, 2011. **94**(1): p. 270-7.
36. Li, K., et al., *Associations of dietary calcium intake and calcium supplementation with myocardial infarction and stroke risk and overall cardiovascular mortality in the Heidelberg cohort of the European Prospective Investigation into Cancer and Nutrition study (EPIC-Heidelberg).* Heart, 2012. **98**(12): p. 920-5.
37. Tang, B.M., et al., *Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis.* Lancet, 2007. **370**(9588): p. 657-66.

38. Cummings, S.R., et al., *Risk factors for hip fracture in white women. Study of Osteoporotic Fractures Research Group.* N Engl J Med, 1995. **332**(12): p. 767-73.
39. Cumming, R.G., et al., *Calcium intake and fracture risk: results from the study of osteoporotic fractures.* Am J Epidemiol, 1997. **145**(10): p. 926-34.
40. Feskanich, D., W.C. Willett, and G.A. Colditz, *Calcium, vitamin D, milk consumption, and hip fractures: a prospective study among postmenopausal women.* Am J Clin Nutr, 2003. **77**(2): p. 504-11.
41. Naveh-Many, T. and J. Silver, *Regulation of parathyroid hormone gene expression by hypocalcemia, hypercalcemia, and vitamin D in the rat.* J Clin Invest, 1990. **86**(4): p. 1313-9.
42. Li, Y.C., et al., *Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice.* Endocrinology, 1998. **139**(10): p. 4391-6.
43. Silver, J., J. Russell, and L.M. Sherwood, *Regulation by vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells.* Proc Natl Acad Sci U S A, 1985. **82**(12): p. 4270-3.
44. Moallem, E., et al., *RNA-Protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate.* J Biol Chem, 1998. **273**(9): p. 5253-9.
45. Gallagher, J.C., et al., *Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients: effect of age and dietary calcium.* J Clin Invest, 1979. **64**(3): p. 729-36.
46. Ireland, P. and J.S. Fordtran, *Effect of dietary calcium and age on jejunal calcium absorption in humans studied by intestinal perfusion.* J Clin Invest, 1973. **52**(11): p. 2672-81.
47. Suda, T., et al., *Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families.* Endocr Rev, 1999. **20**(3): p. 345-57.
48. Favus M. J. and D. Goltzman, *Regulation of Calcium and Magnesium*, in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolsim*, C.J. Rosen 2008, American Society for Bone and Mineral Research: Washington DC.
49. Itoh, R. and Y. Suyama, *Sodium excretion in relation to calcium and hydroxyproline excretion in a healthy Japanese population.* Am J Clin Nutr, 1996. **63**(5): p. 735-40.
50. Kerstetter, J.E., K.O. O'Brien, and K.L. Insogna, *Dietary protein, calcium metabolism, and skeletal homeostasis revisited.* Am J Clin Nutr, 2003. **78**(3 Suppl): p. 584S-592S.
51. Spiegel, D.M. and K. Brady, *Calcium balance in normal individuals and in patients with chronic kidney disease on low- and high-calcium diets.* Kidney Int, 2012. **81**(11): p. 1116-22.
52. Steggerda, F.R. and H.H. Mitchell, *Variability in the calcium metabolism and calcium requirements of adult human subjects.* J Nutr, 1946. **31**: p. 407-22.
53. Steggerda, F.R. and H.H. Mitchell, *The calcium balance of adult human subjects on high- and low-fat (butter) diets.* J Nutr, 1951. **45**(2): p. 201-11.
54. Malm, O.J., *Calcium requirement and adaptation in adult men.* Scand J Clin Lab Invest, 1958. **10 Supp 36**: p. 1-290.
55. Heaney, R.P. and R.R. Recker, *Effects of nitrogen, phosphorus, and caffeine on calcium balance in women.* J Lab Clin Med, 1982. **99**(1): p. 46-55.
56. Spencer, H., et al., *Effect of phosphorus on the absorption of calcium and on the calcium balance in man.* J Nutr, 1978. **108**(3): p. 447-57.

57. Hegsted, M., et al., *Urinary calcium and calcium balance in young men as affected by level of protein and phosphorus intake.* J Nutr, 1981. **111**(3): p. 553-62.
58. Hunt, C.D. and L.K. Johnson, *Calcium requirements: new estimations for men and women by cross-sectional statistical analyses of calcium balance data from metabolic studies.* Am J Clin Nutr, 2007. **86**(4): p. 1054-63.
59. Dawson-Hughes, B., et al., *Effect of lowering dietary calcium intake on fractional whole body calcium retention.* J Clin Endocrinol Metab, 1988. **67**(1): p. 62-8.
60. Norman, D.A., et al., *Jejunal and ileal adaptation to alterations in dietary calcium: changes in calcium and magnesium absorption and pathogenetic role of parathyroid hormone and 1,25-dihydroxyvitamin D.* J Clin Invest, 1981. **67**(6): p. 1599-603.
61. Heaney, R.P., *The bone-remodeling transient: implications for the interpretation of clinical studies of bone mass change.* J Bone Miner Res, 1994. **9**(10): p. 1515-23.
62. Reid, I.R., et al., *Randomized controlled trial of calcium supplementation in healthy, nonosteoporotic, older men.* Arch Intern Med, 2008. **168**(20): p. 2276-82.
63. Ma, J., R.A. Johns, and R.S. Stafford, *Americans are not meeting current calcium recommendations.* Am J Clin Nutr, 2007. **85**(5): p. 1361-6.
64. Pasco, J.A., et al., *Calcium intakes among Australian women: Geelong Osteoporosis Study.* Aust N Z J Med, 2000. **30**(1): p. 21-7.
65. Gass, M. and B. Dawson-Hughes, *Preventing osteoporosis-related fractures: an overview.* Am J Med, 2006. **119**(4 Suppl 1): p. S3-S11.
66. Boonen, S., et al., *The need for clinical guidance in the use of calcium and vitamin D in the management of osteoporosis: a consensus report.* Osteoporos Int, 2004. **15**(7): p. 511-9.
67. Heaney, R.P., *Calcium, dairy products and osteoporosis.* J Am Coll Nutr, 2000. **19**(2 Suppl): p. 83S-99S.
68. Deroisy, R., et al., *Acute changes in serum calcium and parathyroid hormone circulating levels induced by the oral intake of five currently available calcium salts in healthy male volunteers.* Clin Rheumatol, 1997. **16**(3): p. 249-53.
69. Heaney, R.P., et al., *Absorbability and cost effectiveness in calcium supplementation.* J Am Coll Nutr, 2001. **20**(3): p. 239-46.
70. Karp, H.J., M.E. Ketola, and C.J. Lamberg-Allardt, *Acute effects of calcium carbonate, calcium citrate and potassium citrate on markers of calcium and bone metabolism in young women.* Br J Nutr, 2009. **102**(9): p. 1341-7.
71. Reid, I.R., et al., *The acute biochemical effects of four proprietary calcium preparations.* Aust N Z J Med, 1986. **16**(2): p. 193-7.
72. Bischoff-Ferrari, H.A., et al., *Calcium intake and hip fracture risk in men and women: a meta-analysis of prospective cohort studies and randomized controlled trials.* Am J Clin Nutr, 2007. **86**(6): p. 1780-90.
73. Yan, L., et al., *Epidemiological study of hip fracture in Shenyang, People's Republic of China.* Bone, 1999. **24**(2): p. 151-5.
74. Adebajo, A.O., C. Cooper, and J.G. Evans, *Fractures of the hip and distal forearm in West Africa and the United Kingdom.* Age Ageing, 1991. **20**(6): p. 435-8.
75. World Health Organization Collaborating Centre for Metabolic Bone Diseases University of Sheffield. *FRAX World Health Organisation Fracture Assessment Tool.* 2014 [cited 2014 07 July]; Available from: http://www.shef.ac.uk/FRAX/index.aspx.
76. Garvan Institute. *Fracture risk calculator.* 2014 [cited 2014 07 July]; Available from: http://garvan.org.au/promotions/bone-fracture-risk/calculator/.
77. Warensjo, E., et al., *Dietary calcium intake and risk of fracture and osteoporosis: prospective longitudinal cohort study.* BMJ, 2011. **342**: p. d1473.

78. Samelson, E.J., et al., *Calcium intake is not associated with increased coronary artery calcification: the Framingham Study.* Am J Clin Nutr, 2012. **96**(6): p. 1274-80.
79. Anderson, J.J., K.J. Roggenkamp, and C.M. Suchindran, *Calcium intakes and femoral and lumbar bone density of elderly U.S. men and women: National Health and Nutrition Examination Survey 2005-2006 analysis.* J Clin Endocrinol Metab, 2012. **97**(12): p. 4531-9.
80. Ranganathan, R., et al., *The nutritional impact of dairy product consumption on dietary intakes of adults (1995-1996): the Bogalusa Heart Study.* J Am Diet Assoc, 2005. **105**(9): p. 1391-400.
81. Barr, S.I., et al., *Effects of increased consumption of fluid milk on energy and nutrient intake, body weight, and cardiovascular risk factors in healthy older adults.* J Am Diet Assoc, 2000. **100**(7): p. 810-7.
82. Hu, J.F., et al., *Dietary calcium and bone density among middle-aged and elderly women in China.* Am J Clin Nutr, 1993. **58**(2): p. 219-27.
83. Riggs, B.L., et al., *Dietary calcium intake and rates of bone loss in women.* J Clin Invest, 1987. **80**(4): p. 979-82.
84. van Beresteijn, E.C., et al., *Habitual dietary calcium intake and cortical bone loss in perimenopausal women: a longitudinal study.* Calcif Tissue Int, 1990. **47**(6): p. 338-44.
85. Hosking, D.J., et al., *Evidence that increased calcium intake does not prevent early postmenopausal bone loss.* Clin Ther, 1998. **20**(5): p. 933-44.
86. Dennison, E., et al., *Determinants of bone loss in elderly men and women: a prospective population-based study.* Osteoporos Int, 1999. **10**(5): p. 384-91.
87. Hannan, M.T., et al., *Risk factors for longitudinal bone loss in elderly men and women: the Framingham Osteoporosis Study.* J Bone Miner Res, 2000. **15**(4): p. 710-20.
88. Reid, I.R., et al., *Determinants of the rate of bone loss in normal postmenopausal women.* J Clin Endocrinol Metab, 1994. **79**(4): p. 950-4.
89. Uusi-Rasi, K., et al., *Influence of calcium intake and physical activity on proximal femur bone mass and structure among pre- and postmenopausal women. A 10-year prospective study.* Calcif Tissue Int, 2008. **82**(3): p. 171-81.
90. Hansen, M.A., et al., *Potential risk factors for development of postmenopausal osteoporosis--examined over a 12-year period.* Osteoporos Int, 1991. **1**(2): p. 95-102.
91. Nakamura, K., et al., *Effect of low-dose calcium supplements on bone loss in perimenopausal and postmenopausal Asian women: a randomized controlled trial.* J Bone Miner Res, 2012. **27**(11): p. 2264-70.
92. Macdonald, H.M., et al., *Nutritional associations with bone loss during the menopausal transition: evidence of a beneficial effect of calcium, alcohol, and fruit and vegetable nutrients and of a detrimental effect of fatty acids.* Am J Clin Nutr, 2004. **79**(1): p. 155-65.
93. Ho, S.C., et al., *High habitual calcium intake attenuates bone loss in early postmenopausal Chinese women: an 18-month follow-up study.* J Clin Endocrinol Metab, 2004. **89**(5): p. 2166-70.
94. Burger, H., et al., *Risk factors for increased bone loss in an elderly population: the Rotterdam Study.* Am J Epidemiol, 1998. **147**(9): p. 871-9.
95. Sirola, J., et al., *Smoking may impair the bone protective effects of nutritional calcium: a population-based approach.* J Bone Miner Res, 2003. **18**(6): p. 1036-42.
96. Dawson-Hughes, B., P. Jacques, and C. Shipp, *Dietary calcium intake and bone loss from the spine in healthy postmenopausal women.* Am J Clin Nutr, 1987. **46**(4): p. 685-7.

97. Aptel, I., A. Cance-Rouzaud, and H. Grandjean, *Association between calcium ingested from drinking water and femoral bone density in elderly women: evidence from the EPIDOS cohort.* J Bone Miner Res, 1999. **14**(5): p. 829-33.
98. Cauley, J.A., et al., *Factors associated with the lumbar spine and proximal femur bone mineral density in older men.* Osteoporos Int, 2005. **16**(12): p. 1525-37.
99. Bauer, D.C., et al., *Factors associated with appendicular bone mass in older women. The Study of Osteoporotic Fractures Research Group.* Ann Intern Med, 1993. **118**(9): p. 657-65.
100. Kroger, H., et al., *Bone mineral density and risk factors for osteoporosis--a population-based study of 1600 perimenopausal women.* Calcif Tissue Int, 1994. **55**(1): p. 1-7.
101. McCabe, L.D., et al., *Dairy intakes affect bone density in the elderly.* Am J Clin Nutr, 2004. **80**(4): p. 1066-74.
102. Nguyen, T.V., J.R. Center, and J.A. Eisman, *Osteoporosis in elderly men and women: effects of dietary calcium, physical activity, and body mass index.* J Bone Miner Res, 2000. **15**(2): p. 322-31.
103. Korpelainen, R., et al., *Lifestyle factors are associated with osteoporosis in lean women but not in normal and overweight women: a population-based cohort study of 1222 women.* Osteoporos Int, 2003. **14**(1): p. 34-43.
104. Varenna, M., et al., *Effects of dietary calcium intake on body weight and prevalence of osteoporosis in early postmenopausal women.* Am J Clin Nutr, 2007. **86**(3): p. 639-44.
105. Mavroeidi, A., et al., *Physical activity and dietary calcium interactions in bone mass in Scottish postmenopausal women.* Osteoporos Int, 2009. **20**(3): p. 409-16.
106. Devine, A., et al., *Physical activity and calcium consumption are important determinants of lower limb bone mass in older women.* J Bone Miner Res, 2004. **19**(10): p. 1634-9.
107. Bischoff-Ferrari, H.A., et al., *Dietary calcium and serum 25-hydroxyvitamin D status in relation to BMD among U.S. adults.* J Bone Miner Res, 2009. **24**(5): p. 935-42.
108. Yano, K., et al., *The relationship between diet and bone mineral content of multiple skeletal sites in elderly Japanese-American men and women living in Hawaii.* Am J Clin Nutr, 1985. **42**(5): p. 877-88.
109. Pongchaiyakul, C., et al., *Effects of physical activity and dietary calcium intake on bone mineral density and osteoporosis risk in a rural Thai population.* Osteoporos Int, 2004. **15**(10): p. 807-13.
110. Pongchaiyakul, C., et al., *The association of dietary calcium, bone mineral density and biochemical bone turnover markers in rural Thai women.* J Med Assoc Thai, 2008. **91**(3): p. 295-302.
111. Khoo, C.C., et al., *Determinants of bone mineral density in older postmenopausal Chinese women.* Climacteric, 2011. **14**(3): p. 378-83.
112. Cheung, E.Y., et al., *Determinants of bone mineral density in Chinese men.* Osteoporos Int, 2005. **16**(12): p. 1481-6.
113. Lau, E.M., et al., *The determinants of bone mineral density in Chinese men--results from Mr. Os (Hong Kong), the first cohort study on osteoporosis in Asian men.* Osteoporos Int, 2006. **17**(2): p. 297-303.
114. Nieves, J.W., et al., *Calcium and vitamin D intake influence bone mass, but not short-term fracture risk, in Caucasian postmenopausal women from the National Osteoporosis Risk Assessment (NORA) study.* Osteoporos Int, 2008. **19**(5): p. 673-9.

115. Daly, R.M., et al., *Calcium- and vitamin D3-fortified milk reduces bone loss at clinically relevant skeletal sites in older men: a 2-year randomized controlled trial.* J Bone Miner Res, 2006. **21**(3): p. 397-405.
116. Nelson, M.E., et al., *A 1-y walking program and increased dietary calcium in postmenopausal women: effects on bone.* Am J Clin Nutr, 1991. **53**(5): p. 1304-11.
117. Kukuljan, S., et al., *Effects of a multi-component exercise program and calcium-vitamin-D3-fortified milk on bone mineral density in older men: a randomised controlled trial.* Osteoporos Int, 2009. **20**(7): p. 1241-51.
118. Chee, W.S., et al., *The effect of milk supplementation on bone mineral density in postmenopausal Chinese women in Malaysia.* Osteoporos Int, 2003. **14**(10): p. 828-34.
119. Lau, E.M., et al., *Milk supplementation of the diet of postmenopausal Chinese women on a low calcium intake retards bone loss.* J Bone Miner Res, 2001. **16**(9): p. 1704-9.
120. Gui, J.C., et al., *Bone mineral density in postmenopausal Chinese women treated with calcium fortification in soymilk and cow's milk.* Osteoporos Int, 2012. **23**(5): p. 1563-70.
121. Recker, R.R. and R.P. Heaney, *The effect of milk supplements on calcium metabolism, bone metabolism and calcium balance.* Am J Clin Nutr, 1985. **41**(2): p. 254-63.
122. Baran, D., et al., *Dietary modification with dairy products for preventing vertebral bone loss in premenopausal women: a three-year prospective study.* J Clin Endocrinol Metab, 1990. **70**(1): p. 264-70.
123. Prince, R., et al., *The effects of calcium supplementation (milk powder or tablets) and exercise on bone density in postmenopausal women.* J Bone Miner Res, 1995. **10**(7): p. 1068-75.
124. Wickham, C.A., et al., *Dietary calcium, physical activity, and risk of hip fracture: a prospective study.* BMJ, 1989. **299**(6704): p. 889-92.
125. Paganini-Hill, A., et al., *Exercise and other factors in the prevention of hip fracture: the Leisure World study.* Epidemiology, 1991. **2**(1): p. 16-25.
126. Meyer, H.E., et al., *Dietary factors and the incidence of hip fracture in middle-aged Norwegians. A prospective study.* Am J Epidemiol, 1997. **145**(2): p. 117-23.
127. Benetou, V., et al., *Diet and hip fractures among elderly Europeans in the EPIC cohort.* Eur J Clin Nutr, 2011. **65**(1): p. 132-9.
128. Michaelsson, K., et al., *Dietary calcium and vitamin D intake in relation to osteoporotic fracture risk.* Bone, 2003. **32**(6): p. 694-703.
129. Owusu, W., et al., *Calcium intake and the incidence of forearm and hip fractures among men.* J Nutr, 1997. **127**(9): p. 1782-7.
130. Holbrook, T.L., E. Barrett-Connor, and D.L. Wingard, *Dietary calcium and risk of hip fracture: 14-year prospective population study.* Lancet, 1988. **2**(8619): p. 1046-9.
131. Looker, A.C., et al., *Dietary calcium and hip fracture risk: the NHANES I Epidemiologic Follow-Up Study.* Osteoporos Int, 1993. **3**(4): p. 177-84.
132. Bischoff-Ferrari, H.A., et al., *Milk intake and risk of hip fracture in men and women: a meta-analysis of prospective cohort studies.* J Bone Miner Res, 2011. **26**(4): p. 833-9.
133. Lips, P., et al., *An international comparison of serum 25-hydroxyvitamin D measurements.* Osteoporos Int, 1999. **9**(5): p. 394-7.
134. El-Desouki, M.I., S.M. Othman, and M.A. Fouda, *Bone mineral density and bone scintigraphy in adult Saudi female patients with osteomalacia.* Saudi Med J, 2004. **25**(3): p. 355-8.

135. Chapuy, M.C., et al., *Combined calcium and vitamin D3 supplementation in elderly women: confirmation of reversal of secondary hyperparathyroidism and hip fracture risk: the Decalyos II study.* Osteoporos Int, 2002. **13**(3): p. 257-64.
136. Chailurkit, L.O., et al., *Discrepant influence of vitamin D status on parathyroid hormone and bone mass after two years of calcium supplementation.* Clin Endocrinol (Oxf), 2010. **73**(2): p. 167-72.
137. Reid, I.R., et al., *Randomized controlled trial of calcium in healthy older women.* Am J Med, 2006. **119**(9): p. 777-85.
138. Reid, I.R. and M.J. Bolland, *Calcium risk-benefit updated--new WHI analyses.* Maturitas, 2014. **77**(1): p. 1-3.
139. Steingrimsdottir, L., et al., *Relationship between serum parathyroid hormone levels, vitamin D sufficiency, and calcium intake.* JAMA, 2005. **294**(18): p. 2336-41.
140. Karkkainen, M.U., J.W. Wiersma, and C.J. Lamberg-Allardt, *Postprandial parathyroid hormone response to four calcium-rich foodstuffs.* Am J Clin Nutr, 1997. **65**(6): p. 1726-30.
141. Talbot, J.R., et al., *Calcium bioavailability and parathyroid hormone acute changes after oral intake of dairy and nondairy products in healthy volunteers.* Osteoporos Int, 1999. **10**(2): p. 137-42.
142. Heaney, R.P., C.M. Weaver, and R.R. Recker, *Calcium absorbability from spinach.* Am J Clin Nutr, 1988. **47**(4): p. 707-9.
143. Heaney, R.P., C.M. Weaver, and M.L. Fitzsimmons, *Soybean phytate content: effect on calcium absorption.* Am J Clin Nutr, 1991. **53**(3): p. 745-7.
144. Chan, G.M., K. Hoffman, and M. McMurry, *Effects of dairy products on bone and body composition in pubertal girls.* J Pediatr, 1995. **126**(4): p. 551-6.
145. Kerstetter, J.E., et al., *The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women.* J Clin Endocrinol Metab, 2005. **90**(1): p. 26-31.
146. Karp, H.J., et al., *Acute effects of different phosphorus sources on calcium and bone metabolism in young women: a whole-foods approach.* Calcif Tissue Int, 2007. **80**(4): p. 251-8.
147. Cornish, J., et al., *Lactoferrin is a potent regulator of bone cell activity and increases bone formation in vivo.* Endocrinology, 2004. **145**(9): p. 4366-74.
148. Cornish, J., et al., *Modulation of osteoclastogenesis by fatty acids.* Endocrinology, 2008. **149**(11): p. 5688-95.
149. Reid, I.R., et al., *Calcium supplementation: balancing the cardiovascular risks.* Maturitas, 2011. **69**(4): p. 289-95.
150. Birkett, N.J., *Comments on a meta-analysis of the relation between dietary calcium intake and blood pressure.* Am J Epidemiol, 1998. **148**(3): p. 223-8; discussion 232-3.
151. Jacqmain, M., et al., *Calcium intake, body composition, and lipoprotein-lipid concentrations in adults.* Am J Clin Nutr, 2003. **77**(6): p. 1448-52.
152. Davies, K.M., et al., *Calcium intake and body weight.* J Clin Endocrinol Metab, 2000. **85**(12): p. 4635-8.
153. Reid, I.R., et al., *Effects of calcium supplementation on lipids, blood pressure, and body composition in healthy older men: a randomized controlled trial.* Am J Clin Nutr, 2010. **91**(1): p. 131-9.
154. Griffith, L.E., et al., *The influence of dietary and nondietary calcium supplementation on blood pressure: an updated metaanalysis of randomized controlled trials.* Am J Hypertens, 1999. **12**(1 Pt 1): p. 84-92.
155. Dickinson, H.O., et al., *Calcium supplementation for the management of primary hypertension in adults.* Cochrane Database Syst Rev, 2006(2): p. CD004639.

156. van Mierlo, L.A., et al., *Blood pressure response to calcium supplementation: a meta-analysis of randomized controlled trials.* J Hum Hypertens, 2006. **20**(8): p. 571-80.
157. Reid, I.R., et al., *Effects of calcium supplementation on serum lipid concentrations in normal older women: a randomized controlled trial.* Am J Med, 2002. **112**(5): p. 343-7.
158. Barr, S.I., *Increased dairy product or calcium intake: is body weight or composition affected in humans?* J Nutr, 2003. **133**(1): p. 245S-248S.
159. Reid, I.R., et al., *Effects of calcium supplementation on body weight and blood pressure in normal older women: a randomized controlled trial.* J Clin Endocrinol Metab, 2005. **90**(7): p. 3824-9.
160. Block, G.A., et al., *Mortality effect of coronary calcification and phosphate binder choice in incident hemodialysis patients.* Kidney Int, 2007. **71**(5): p. 438-41.
161. Braun, J., et al., *Long-term comparison of a calcium-free phosphate binder and calcium carbonate--phosphorus metabolism and cardiovascular calcification.* Clin Nephrol, 2004. **62**(2): p. 104-15.
162. Block, G.A., et al., *Effects of sevelamer and calcium on coronary artery calcification in patients new to hemodialysis.* Kidney Int, 2005. **68**(4): p. 1815-24.
163. Bolland, M.J., et al., *Vascular events in healthy older women receiving calcium supplementation: randomised controlled trial.* BMJ, 2008. **336**(7638): p. 262-6.
164. Bolland, M.J., et al., *Effect of calcium supplements on risk of myocardial infarction and cardiovascular events: meta-analysis.* BMJ, 2010. **341**: p. c3691.
165. Hsia, J., et al., *Calcium/vitamin D supplementation and cardiovascular events.* Circulation, 2007. **115**(7): p. 846-54.
166. Lewis, J.R., et al., *Calcium supplementation and the risks of atherosclerotic vascular disease in older women: results of a 5-year RCT and a 4.5-year follow-up.* J Bone Miner Res, 2011. **26**(1): p. 35-41.
167. Sambrook, P.N., et al., *Does increased sunlight exposure work as a strategy to improve vitamin D status in the elderly: a cluster randomised controlled trial.* Osteoporos Int, 2012. **23**(2): p. 615-24.
168. Million Women Study Collaborators, *Patterns of use of hormone replacement therapy in one million women in Britain, 1996-2000.* BJOG, 2002. **109**(12): p. 1319-30.
169. Pentti, K., et al., *Use of calcium supplements and the risk of coronary heart disease in 52-62-year-old women: The Kuopio Osteoporosis Risk Factor and Prevention Study.* Maturitas, 2009. **63**(1): p. 73-8.
170. Xiao, Q., et al., *Dietary and supplemental calcium intake and cardiovascular disease mortality: the National Institutes of Health-AARP diet and health study.* JAMA Intern Med, 2013. **173**(8): p. 639-46.
171. Michaelsson, K., et al., *Long term calcium intake and rates of all cause and cardiovascular mortality: community based prospective longitudinal cohort study.* BMJ, 2013. **346**: p. f228.
172. Van Hemelrijck, M., et al., *Calcium intake and serum concentration in relation to risk of cardiovascular death in NHANES III.* PLoS One, 2013. **8**(4): p. e61037.
173. Bostick, R.M., et al., *Relation of calcium, vitamin D, and dairy food intake to ischemic heart disease mortality among postmenopausal women.* Am J Epidemiol, 1999. **149**(2): p. 151-61.
174. Al-Delaimy, W.K., et al., *A prospective study of calcium intake from diet and supplements and risk of ischemic heart disease among men.* Am J Clin Nutr, 2003. **77**(4): p. 814-8.
175. Umesawa, M., et al., *Dietary intake of calcium in relation to mortality from cardiovascular disease: the JACC Study.* Stroke, 2006. **37**(1): p. 20-6.

176. Umesawa, M., et al., *Dietary calcium intake and risks of stroke, its subtypes, and coronary heart disease in Japanese: the JPHC Study Cohort I.* Stroke, 2008. **39**(9): p. 2449-56.
177. Larsson, S.C., N. Orsini, and A. Wolk, *Dietary calcium intake and risk of stroke: a dose-response meta-analysis.* Am J Clin Nutr, 2013. **97**(5): p. 951-7.
178. Karkkainen, M.U., et al., *Does it make a difference how and when you take your calcium? The acute effects of calcium on calcium and bone metabolism.* Am J Clin Nutr, 2001. **74**(3): p. 335-42.
179. Lind, L., et al., *Serum calcium: a new, independent, prospective risk factor for myocardial infarction in middle-aged men followed for 18 years.* J Clin Epidemiol, 1997. **50**(8): p. 967-73.
180. Jorde, R., et al., *Serum calcium and cardiovascular risk factors and diseases: the Tromso study.* Hypertension, 1999. **34**(3): p. 484-90.
181. Larsson, T.E., et al., *Conjoint effects of serum calcium and phosphate on risk of total, cardiovascular, and noncardiovascular mortality in the community.* Arterioscler Thromb Vasc Biol, 2010. **30**(2): p. 333-9.
182. Slinin, Y., et al., *Serum calcium, phosphorus and cardiovascular events in post-menopausal women.* Int J Cardiol, 2011. **149**(3): p. 335-40.
183. Dhingra, R., et al., *Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community.* Arch Intern Med, 2007. **167**(9): p. 879-85.
184. Iseri, L.T. and J.H. French, *Magnesium: nature's physiologic calcium blocker.* Am Heart J, 1984. **108**(1): p. 188-93.
185. Qu, X., et al., *Magnesium and the risk of cardiovascular events: a meta-analysis of prospective cohort studies.* PLoS One, 2013. **8**(3): p. e57720.
186. Shah, N.P., *Effects of milk-derived bioactives: an overview.* Br J Nutr, 2000. **84 Suppl 1**: p. S3-10.
187. Meisel, H., *Biochemical properties of regulatory peptides derived from milk proteins.* Biopolymers, 1997. **43**(2): p. 119-28.
188. McCarron, D.A., et al., *Blood pressure and nutrient intake in the United States.* Science, 1984. **224**(4656): p. 1392-8.
189. Morikawa, Y., et al., *A cross-sectional study on association of calcium intake with blood pressure in Japanese population.* J Hum Hypertens, 2002. **16**(2): p. 105-10.
190. Schroder, H., E. Schmelz, and J. Marrugat, *Relationship between diet and blood pressure in a representative Mediterranean population.* Eur J Nutr, 2002. **41**(4): p. 161-7.
191. Hajjar, I.M., C.E. Grim, and T.A. Kotchen, *Dietary calcium lowers the age-related rise in blood pressure in the United States: the NHANES III survey.* J Clin Hypertens (Greenwich), 2003. **5**(2): p. 122-6.
192. Townsend, M.S., et al., *Low mineral intake is associated with high systolic blood pressure in the Third and Fourth National Health and Nutrition Examination Surveys: could we all be right?* Am J Hypertens, 2005. **18**(2 Pt 1): p. 261-9.
193. Margolis, K.L., et al., *Effect of calcium and vitamin D supplementation on blood pressure: the Women's Health Initiative Randomized Trial.* Hypertension, 2008. **52**(5): p. 847-55.
194. Nilsson, I.L., et al., *Endothelial vasodilatory function and blood pressure response to local and systemic hypercalcemia.* Surgery, 2001. **130**(6): p. 986-90.
195. Kamycheva, E., et al., *Effects of acute hypercalcaemia on blood pressure in subjects with and without parathyroid hormone secretion.* Acta Physiol Scand, 2005. **184**(2): p. 113-9.

196. Aoki, K. and K. Miyagawa, *Correlation of increased serum calcium with elevated blood pressure and vascular resistance during calcium infusion in normotensive man.* J Hypertens, 1990. **8**(6): p. 579-83.
197. Suzuki, T. and K. Aoki, *Hypertensive effects of calcium infusion in subjects with normotension and hypertension.* J Hypertens, 1988. **6**(12): p. 1003-8.
198. Ellison, D.H., et al., *Effects of calcium infusion on blood pressure in hypertensive and normotensive humans.* Hypertension, 1986. **8**(6): p. 497-505.
199. Berl, T., et al., *Mechanism of acute hypercalcemic hypertension in the conscious rat.* Hypertension, 1985. **7**(6 Pt 1): p. 923-30.
200. Gabutti, L., et al., *Haemodynamic consequences of changing bicarbonate and calcium concentrations in haemodialysis fluids.* Nephrol Dial Transplant, 2009. **24**(3): p. 973-81.
201. Maynard, J.C., et al., *Blood pressure response to changes in serum ionized calcium during hemodialysis.* Ann Intern Med, 1986. **104**(3): p. 358-61.
202. van der Sande, F.M., et al., *Effect of dialysate calcium concentrations on intradialytic blood pressure course in cardiac-compromised patients.* Am J Kidney Dis, 1998. **32**(1): p. 125-31.
203. van Kuijk, W.H., et al., *Influence of changes in ionized calcium on cardiovascular reactivity during hemodialysis.* Clin Nephrol, 1997. **47**(3): p. 190-6.
204. Fellner, S.K., et al., *Physiological mechanisms for calcium-induced changes in systemic arterial pressure in stable dialysis patients.* Hypertension, 1989. **13**(3): p. 213-8.
205. Henrich, W.L., J.M. Hunt, and J.V. Nixon, *Increased ionized calcium and left ventricular contractility during hemodialysis.* N Engl J Med, 1984. **310**(1): p. 19-23.
206. LeBeouf, A., et al., *Effects of acute variation of dialysate calcium concentrations on arterial stiffness and aortic pressure waveform.* Nephrol Dial Transplant, 2009. **24**(12): p. 3788-94.
207. Burt, M.G., et al., *Acute effect of calcium citrate on serum calcium and cardiovascular function.* J Bone Miner Res, 2013. **28**(2): p. 412-8.
208. Yaron, M., et al., *Effects of a typical acute oral calcium load on arterial properties and endothelial function in healthy subjects.* Eur J Clin Nutr, 2014. **68**(5): p. 608-12.
209. Mann, K.G., *Biochemistry and physiology of blood coagulation.* Thromb Haemost, 1999. **82**(2): p. 165-74.
210. House, M.G., et al., *Expression of an extracellular calcium-sensing receptor in human and mouse bone marrow cells.* J Bone Miner Res, 1997. **12**(12): p. 1959-70.
211. Ataullakhanov, F.I., et al., *Calcium threshold in human plasma clotting kinetics.* Thromb Res, 1994. **75**(4): p. 383-94.
212. Mallett, S.V. and D.J. Cox, *Thrombelastography.* Br J Anaesth, 1992. **69**(3): p. 307-13.
213. Haemoscope Corporation, *TEG Haemostasis Analyzer TEG 5000 User's Manual* ed. Haemoscope Corporation. 2006, Niles IL.
214. Perry, D.J., et al., *Point-of-care testing in haemostasis.* Br J Haematol, 2010. **150**(5): p. 501-14.
215. O'Donnell, J., et al., *Role of the Thrombelastograph as an adjunctive test in thrombophilia screening.* Blood Coagul Fibrinolysis, 2004. **15**(3): p. 207-11.
216. Hvitfeldt Poulsen, L., et al., *Whole blood thrombelastographic coagulation profiles using minimal tissue factor activation can display hypercoagulation in thrombosis-prone patients.* Scand J Clin Lab Invest, 2006. **66**(4): p. 329-36.

217. McCrath, D.J., et al., *Thromboelastography maximum amplitude predicts postoperative thrombotic complications including myocardial infarction.* Anesth Analg, 2005. **100**(6): p. 1576-83.
218. Hilgard, P., *Experimental hypercalcaemia and whole blood clotting.* J Clin Pathol, 1973. **26**(8): p. 616-9.
219. James, M.F. and A.M. Roche, *Dose-response relationship between plasma ionized calcium concentration and thrombelastography.* J Cardiothorac Vasc Anesth, 2004. **18**(5): p. 581-6.
220. Rajwal, S., M. Richards, and M. O'Meara, *The use of recalcified citrated whole blood -- a pragmatic approach for thromboelastography in children.* Paediatr Anaesth, 2004. **14**(8): p. 656-60.
221. Roche, A.M., et al., *Citrated blood does not reliably reflect fresh whole blood coagulability in trials of in vitro hemodilution.* Anesth Analg, 2003. **96**(1): p. 58-61, table of contents.
222. Wang, T.K., et al., *Relationships between vascular calcification, calcium metabolism, bone density, and fractures.* J Bone Miner Res, 2010. **25**(12): p. 2777-85.
223. Rubin, M.R., et al., *Carotid artery plaque thickness is associated with increased serum calcium levels: the Northern Manhattan study.* Atherosclerosis, 2007. **194**(2): p. 426-32.
224. Lewis, J.R., et al., *The effects of 3 years of calcium supplementation on common carotid artery intimal medial thickness and carotid atherosclerosis in older women: an ancillary study of the CAIFOS randomized controlled trial.* J Bone Miner Res, 2014. **29**(3): p. 534-41.
225. Pasch, A., et al., *Nanoparticle-based test measures overall propensity for calcification in serum.* J Am Soc Nephrol, 2012. **23**(10): p. 1744-52.
226. Heiss, A., et al., *Structural basis of calcification inhibition by alpha 2-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles.* J Biol Chem, 2003. **278**(15): p. 13333-41.
227. Smith, E.R., et al., *Serum Calcification Propensity Predicts All-Cause Mortality in Predialysis CKD.* J Am Soc Nephrol, 2013.
228. Shanahan, C.M., et al., *Arterial calcification in chronic kidney disease: key roles for calcium and phosphate.* Circ Res, 2011. **109**(6): p. 697-711.
229. Schafer, C., et al., *The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification.* J Clin Invest, 2003. **112**(3): p. 357-66.
230. Ketteler, M., et al., *Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study.* Lancet, 2003. **361**(9360): p. 827-33.
231. Hermans, M.M., et al., *Association of serum fetuin-A levels with mortality in dialysis patients.* Kidney Int, 2007. **72**(2): p. 202-7.
232. Mehrotra, R., et al., *Serum fetuin-A in nondialyzed patients with diabetic nephropathy: relationship with coronary artery calcification.* Kidney Int, 2005. **67**(3): p. 1070-7.
233. Weikert, C., et al., *Plasma fetuin-a levels and the risk of myocardial infarction and ischemic stroke.* Circulation, 2008. **118**(24): p. 2555-62.
234. Manenti, L., A. Vaglio, and S. Pasquali, *Increased fetuin-A levels following treatment with a vitamin D analog.* Kidney Int, 2010. **78**(11): p. 1187; author reply 1187-9.
235. Schaible, J., et al., *Serum fetuin-A and vitamin D in children with mild-to-severe chronic kidney disease: a cross-sectional study.* Nephrol Dial Transplant, 2012. **27**(3): p. 1107-13.

236. Meyer, J.L., *Can biological calcification occur in the presence of pyrophosphate?* Arch Biochem Biophys, 1984. **231**(1): p. 1-8.
237. O'Neill, W.C., M.K. Sigrist, and C.W. McIntyre, *Plasma pyrophosphate and vascular calcification in chronic kidney disease.* Nephrol Dial Transplant, 2010. **25**(1): p. 187-91.
238. Rachow, J.W. and L.M. Ryan, *Inorganic pyrophosphate metabolism in arthritis.* Rheum Dis Clin North Am, 1988. **14**(2): p. 289-302.
239. Lomashvili, K.A., W. Khawandi, and W.C. O'Neill, *Reduced plasma pyrophosphate levels in hemodialysis patients.* J Am Soc Nephrol, 2005. **16**(8): p. 2495-500.
240. Seiler, S., et al., *FGF-23 and future cardiovascular events in patients with chronic kidney disease before initiation of dialysis treatment.* Nephrol Dial Transplant, 2010. **25**(12): p. 3983-9.
241. Kendrick, J., et al., *FGF-23 associates with death, cardiovascular events, and initiation of chronic dialysis.* J Am Soc Nephrol, 2011. **22**(10): p. 1913-22.
242. Nakano, C., et al., *Intact fibroblast growth factor 23 levels predict incident cardiovascular event before but not after the start of dialysis.* Bone, 2012. **50**(6): p. 1266-74.
243. Gutierrez, O.M., et al., *Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis.* N Engl J Med, 2008. **359**(6): p. 584-92.
244. Jean, G., et al., *High levels of serum fibroblast growth factor (FGF)-23 are associated with increased mortality in long haemodialysis patients.* Nephrol Dial Transplant, 2009. **24**(9): p. 2792-6.
245. Mirza, M.A., et al., *Circulating fibroblast growth factor-23 is associated with vascular dysfunction in the community.* Atherosclerosis, 2009. **205**(2): p. 385-90.
246. Mirza, M.A., et al., *Serum intact FGF23 associate with left ventricular mass, hypertrophy and geometry in an elderly population.* Atherosclerosis, 2009. **207**(2): p. 546-51.
247. Mirza, M.A., et al., *Relationship between circulating FGF23 and total body atherosclerosis in the community.* Nephrol Dial Transplant, 2009. **24**(10): p. 3125-31.
248. Gupta, A., et al., *FGF-23 is elevated by chronic hyperphosphatemia.* J Clin Endocrinol Metab, 2004. **89**(9): p. 4489-92.
249. Saito, H., et al., *Circulating FGF-23 is regulated by 1alpha,25-dihydroxyvitamin D3 and phosphorus in vivo.* J Biol Chem, 2005. **280**(4): p. 2543-9.
250. Shimada, T., et al., *Vitamin D receptor-independent FGF23 actions in regulating phosphate and vitamin D metabolism.* Am J Physiol Renal Physiol, 2005. **289**(5): p. F1088-95.
251. Rodriguez-Ortiz, M.E., et al., *Calcium deficiency reduces circulating levels of FGF23.* J Am Soc Nephrol, 2012. **23**(7): p. 1190-7.
252. Garland, C., et al., *Dietary vitamin D and calcium and risk of colorectal cancer: a 19-year prospective study in men.* Lancet, 1985. **1**(8424): p. 307-9.
253. Kampman, E., et al., *Calcium, vitamin D, sunshine exposure, dairy products and colon cancer risk (United States).* Cancer Causes Control, 2000. **11**(5): p. 459-66.
254. Wu, K., et al., *Calcium intake and risk of colon cancer in women and men.* J Natl Cancer Inst, 2002. **94**(6): p. 437-46.
255. Shin, M.H., et al., *Intake of dairy products, calcium, and vitamin d and risk of breast cancer.* J Natl Cancer Inst, 2002. **94**(17): p. 1301-11.
256. McCullough, M.L., et al., *Dairy, calcium, and vitamin D intake and postmenopausal breast cancer risk in the Cancer Prevention Study II Nutrition Cohort.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(12): p. 2898-904.

257. Giovannucci, E., et al., *Calcium and fructose intake in relation to risk of prostate cancer.* Cancer Res, 1998. **58**(3): p. 442-7.
258. Chan, J.M., et al., *Dairy products, calcium, phosphorous, vitamin D, and risk of prostate cancer (Sweden).* Cancer Causes Control, 1998. **9**(6): p. 559-66.
259. Chan, J.M., et al., *Dairy products, calcium, and prostate cancer risk in the Physicians' Health Study.* Am J Clin Nutr, 2001. **74**(4): p. 549-54.
260. Kearney, J., et al., *Calcium, vitamin D, and dairy foods and the occurrence of colon cancer in men.* Am J Epidemiol, 1996. **143**(9): p. 907-17.
261. Martinez, M.E., et al., *Calcium, vitamin D, and the occurrence of colorectal cancer among women.* J Natl Cancer Inst, 1996. **88**(19): p. 1375-82.
262. Larsson, S.C., L. Bergkvist, and A. Wolk, *Long-term dietary calcium intake and breast cancer risk in a prospective cohort of women.* Am J Clin Nutr, 2009. **89**(1): p. 277-82.
263. Park, S.Y., et al., *Calcium, vitamin D, and dairy product intake and prostate cancer risk: the Multiethnic Cohort Study.* Am J Epidemiol, 2007. **166**(11): p. 1259-69.
264. Park, Y., et al., *Calcium, dairy foods, and risk of incident and fatal prostate cancer: the NIH-AARP Diet and Health Study.* Am J Epidemiol, 2007. **166**(11): p. 1270-9.
265. Anderson, L.N., et al., *Vitamin D and calcium intakes and breast cancer risk in pre- and postmenopausal women.* Am J Clin Nutr, 2010. **91**(6): p. 1699-707.
266. Lappe, J.M., et al., *Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial.* Am J Clin Nutr, 2007. **85**(6): p. 1586-91.
267. Baron, J.A., et al., *Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group.* N Engl J Med, 1999. **340**(2): p. 101-7.
268. Bonithon-Kopp, C., et al., *Calcium and fibre supplementation in prevention of colorectal adenoma recurrence: a randomised intervention trial. European Cancer Prevention Organisation Study Group.* Lancet, 2000. **356**(9238): p. 1300-6.
269. Baron, J.A., et al., *Risk of prostate cancer in a randomized clinical trial of calcium supplementation.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(3): p. 586-9.
270. Avenell, A., et al., *Long-term follow-up for mortality and cancer in a randomized placebo-controlled trial of vitamin D(3) and/or calcium (RECORD trial).* J Clin Endocrinol Metab, 2012. **97**(2): p. 614-22.
271. Wactawski-Wende, J., et al., *Calcium plus vitamin D supplementation and the risk of colorectal cancer.* N Engl J Med, 2006. **354**(7): p. 684-96.
272. Chlebowski, R.T., et al., *Calcium plus vitamin D supplementation and the risk of breast cancer.* J Natl Cancer Inst, 2008. **100**(22): p. 1581-91.
273. Tang, J.Y., et al., *Calcium plus vitamin D supplementation and the risk of nonmelanoma and melanoma skin cancer: post hoc analyses of the women's health initiative randomized controlled trial.* J Clin Oncol, 2011. **29**(22): p. 3078-84.
274. Iso, H., et al., *Prospective study of calcium, potassium, and magnesium intake and risk of stroke in women.* Stroke, 1999. **30**(9): p. 1772-9.
275. Martins, I., et al., *Vitamin and mineral intakes in elderly.* J Nutr Health Aging, 2002. **6**(1): p. 63-5.
276. Lovelace, H.Y. and S.I. Barr, *Diagnosis, symptoms, and calcium intakes of individuals with self-reported lactose intolerance.* J Am Coll Nutr, 2005. **24**(1): p. 51-7.
277. Angus, R.M., et al., *A simple method for assessing calcium intake in Caucasian women.* J Am Diet Assoc, 1989. **89**(2): p. 209-14.
278. Horowitz, M., et al., *Oral calcium suppresses biochemical markers of bone resorption in normal men.* Am J Clin Nutr, 1994. **60**(6): p. 965-8.

279. Thomas, S.D., et al., *Suppression of parathyroid hormone and bone resorption by calcium carbonate and calcium citrate in postmenopausal women.* Calcif Tissue Int, 2008. **83**(2): p. 81-4.
280. Buclin, T., A.F. Jacquet, and P. Burckhardt, *[Intestinal absorption of calcium gluconate and oseine-mineral complex: an evaluation by conventional analyses]*. Schweiz Med Wochenschr, 1986. **116**(50): p. 1780-3.
281. Nicar, M.J. and C.Y. Pak, *Calcium bioavailability from calcium carbonate and calcium citrate.* J Clin Endocrinol Metab, 1985. **61**(2): p. 391-3.
282. Harvey, J.A., M.M. Zobitz, and C.Y. Pak, *Dose dependency of calcium absorption: a comparison of calcium carbonate and calcium citrate.* J Bone Miner Res, 1988. **3**(3): p. 253-8.
283. Recker, R.R., *Calcium absorption and achlorhydria.* N Engl J Med, 1985. **313**(2): p. 70-3.
284. Heaney, R.P., R.R. Recker, and C.M. Weaver, *Absorbability of calcium sources: the limited role of solubility.* Calcif Tissue Int, 1990. **46**(5): p. 300-4.
285. Ekman, M., et al., *Comparative absorption of calcium from carbonate tablets, lactogluconate/carbonate effervescent tablet, and chloride solution.* Bone, 1991. **12**(2): p. 93-7.
286. el-Hajj Fuleihan, G., et al., *The parathyroid hormone circadian rhythm is truly endogenous--a general clinical research center study.* J Clin Endocrinol Metab, 1997. **82**(1): p. 281-6.
287. D'Erasmo, E., et al., *Calcium homeostasis during oral glucose load in healthy women.* Horm Metab Res, 1999. **31**(4): p. 271-3.
288. Greenspan, S.L., et al., *Diurnal variation of bone mineral turnover in elderly men and women.* Calcif Tissue Int, 1997. **60**(5): p. 419-23.
289. Wichers, M., et al., *Diurnal rhythm of CrossLaps in human serum.* Clin Chem, 1999. **45**(10): p. 1858-60.
290. Qvist, P., et al., *Circadian variation in the serum concentration of C-terminal telopeptide of type I collagen (serum CTx): effects of gender, age, menopausal status, posture, daylight, serum cortisol, and fasting.* Bone, 2002. **31**(1): p. 57-61.
291. Bjarnason, N.H., et al., *Mechanism of circadian variation in bone resorption.* Bone, 2002. **30**(1): p. 307-13.
292. Henriksen, D.B., et al., *Role of gastrointestinal hormones in postprandial reduction of bone resorption.* J Bone Miner Res, 2003. **18**(12): p. 2180-9.
293. Ahmad, A.M., et al., *Parathyroid hormone secretory pattern, circulating activity, and effect on bone turnover in adult growth hormone deficiency.* Bone, 2003. **32**(2): p. 170-9.
294. Riggs, B.L., et al., *Long-term effects of calcium supplementation on serum parathyroid hormone level, bone turnover, and bone loss in elderly women.* J Bone Miner Res, 1998. **13**(2): p. 168-74.
295. Ruegsegger, P., A. Keller, and M.A. Dambacher, *Comparison of the treatment effects of ossein-hydroxyapatite compound and calcium carbonate in osteoporotic females.* Osteoporos Int, 1995. **5**(1): p. 30-4.
296. Pelayo, I., et al., *Raloxifene plus ossein-hydroxyapatite compound versus raloxifene plus calcium carbonate to control bone loss in postmenopausal women: a randomized trial.* Menopause, 2008. **15**(6): p. 1132-8.
297. Ciria-Recasens, M., et al., *Comparison of the effects of ossein-hydroxyapatite complex and calcium carbonate on bone metabolism in women with senile osteoporosis: a randomized, open-label, parallel-group, controlled, prospective study.* Clin Drug Investig, 2011. **31**(12): p. 817-24.

298. Crook, M.A., V. Hally, and J.V. Panteli, *The importance of the refeeding syndrome.* Nutrition, 2001. **17**(7-8): p. 632-7.
299. Foley, R.N., et al., *Calcium-phosphate levels and cardiovascular disease in community-dwelling adults: the Atherosclerosis Risk in Communities (ARIC) Study.* Am Heart J, 2008. **156**(3): p. 556-63.
300. Foley, R.N., et al., *Serum phosphorus levels associate with coronary atherosclerosis in young adults.* J Am Soc Nephrol, 2009. **20**(2): p. 397-404.
301. Shin, S., et al., *Impact of serum calcium and phosphate on coronary atherosclerosis detected by cardiac computed tomography.* Eur Heart J, 2012. **33**(22): p. 2873-81.
302. Bolland, M.J., et al., *Abdominal aortic calcification on vertebral morphometry images predicts incident myocardial infarction.* J Bone Miner Res, 2010. **25**(3): p. 505-12.
303. Millar-Craig, M.W., C.N. Bishop, and E.B. Raftery, *Circadian variation of blood-pressure.* Lancet, 1978. **1**(8068): p. 795-7.
304. Aronow, W.S. and C. Ahn, *Postprandial hypotension in 499 elderly persons in a long-term health care facility.* J Am Geriatr Soc, 1994. **42**(9): p. 930-2.
305. Luciano, G.L., M.J. Brennan, and M.B. Rothberg, *Postprandial hypotension.* Am J Med, 2010. **123**(3): p. 281 e1-6.
306. Pickering, T.G., et al., *How common is white coat hypertension?* JAMA, 1988. **259**(2): p. 225-8.
307. Li, Z.L., et al., *[Effects of extracellular calcium concentration on platelets aggregation, coagulation indices and thromboelastography]*. Zhonghua Yi Xue Za Zhi, 2010. **90**(22): p. 1547-50.
308. Na, H.S., et al., *Effects of magnesium sulphate on postoperative coagulation, measured by rotational thromboelastometry (ROTEM((R)))*. Anaesthesia, 2012. **67**(8): p. 862-9.
309. Harnett, M.J., S. Datta, and K. Bhavani-Shankar, *The effect of magnesium on coagulation in parturients with preeclampsia.* Anesth Analg, 2001. **92**(5): p. 1257-60.
310. Karon, B.S., *Why is everyone so excited about thromboelastrography (TEG)?* Clin Chim Acta, 2014. **436**: p. 143-8.
311. Burnett, S.M., et al., *Regulation of C-terminal and intact FGF-23 by dietary phosphate in men and women.* J Bone Miner Res, 2006. **21**(8): p. 1187-96.
312. Nishida, Y., et al., *Acute effect of oral phosphate loading on serum fibroblast growth factor 23 levels in healthy men.* Kidney Int, 2006. **70**(12): p. 2141-7.
313. Burckhardt, P., *Potential negative cardiovascular effects of calcium supplements.* Osteoporos Int, 2011. **22**(6): p. 1645-7.
314. Meier, C. and M.E. Kranzlin, *Calcium supplementation, osteoporosis and cardiovascular disease.* Swiss Med Wkly, 2011. **141**: p. w13260.
315. Heaney, R.P., et al., *Calcium fortification systems differ in bioavailability.* J Am Diet Assoc, 2005. **105**(5): p. 807-9.
316. Clowes, J.A., et al., *Effect of feeding on bone turnover markers and its impact on biological variability of measurements.* Bone, 2002. **30**(6): p. 886-90.
317. Licata, A.A., *Acute effects of increased meat protein on urinary electrolytes and cyclic adenosine monophosphate and serum parathyroid hormone.* Am J Clin Nutr, 1981. **34**(9): p. 1779-84.
318. Guillemant, J. and S. Guillemant, *Comparison of the suppressive effect of two doses (500 mg vs 1500 mg) of oral calcium on parathyroid hormone secretion and on urinary cyclic AMP.* Calcif Tissue Int, 1993. **53**(5): p. 304-6.
319. Sheikh, M.S., et al., *Gastrointestinal absorption of calcium from milk and calcium salts.* N Engl J Med, 1987. **317**(9): p. 532-6.

320. Recker, R.R., et al., *Calcium absorbability from milk products, an imitation milk, and calcium carbonate.* Am J Clin Nutr, 1988. **47**(1): p. 93-5.
321. Green, J.H., C. Booth, and R. Bunning, *Postprandial metabolic responses to milk enriched with milk calcium are different from responses to milk enriched with calcium carbonate.* Asia Pac J Clin Nutr, 2003. **12**(1): p. 109-19.
322. Heaney, R.P., et al., *Meal effects on calcium absorption.* Am J Clin Nutr, 1989. **49**(2): p. 372-6.
323. Kestenbaum, B., et al., *Serum phosphate levels and mortality risk among people with chronic kidney disease.* J Am Soc Nephrol, 2005. **16**(2): p. 520-8.
324. Ribeiro, S., et al., *Cardiac valve calcification in haemodialysis patients: role of calcium-phosphate metabolism.* Nephrol Dial Transplant, 1998. **13**(8): p. 2037-40.
325. Block, G.A., et al., *Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study.* Am J Kidney Dis, 1998. **31**(4): p. 607-17.
326. Wang, L., et al., *Dietary intake of dairy products, calcium, and vitamin D and the risk of hypertension in middle-aged and older women.* Hypertension, 2008. **51**(4): p. 1073-9.
327. Patterson, E., et al., *Association between Dairy Food Consumption and Risk of Myocardial Infarction in Women Differs by Type of Dairy Food.* J Nutr, 2013. **143**(1): p. 74-9.
328. Moyer, V.A., *Vitamin D and calcium supplementation to prevent fractures in adults: U.S. Preventive Services Task Force recommendation statement.* Ann Intern Med, 2013. **158**(9): p. 691-6.
329. Rothman, A.J., *Toward a theory-based analysis of behavioral maintenance.* Health Psychol, 2000. **19**(1 Suppl): p. 64-9.
330. Cumming, R.G. and M.C. Nevitt, *Calcium for prevention of osteoporotic fractures in postmenopausal women.* J Bone Miner Res, 1997. **12**(9): p. 1321-9.
331. Jackson, R.D., et al., *Calcium plus vitamin D supplementation and the risk of fractures.* N Engl J Med, 2006. **354**(7): p. 669-83.
332. Prince, R.L., et al., *Effects of calcium supplementation on clinical fracture and bone structure: results of a 5-year, double-blind, placebo-controlled trial in elderly women.* Arch Intern Med, 2006. **166**(8): p. 869-75.
333. Grant, A.M., et al., *Oral vitamin D3 and calcium for secondary prevention of low-trauma fractures in elderly people (Randomised Evaluation of Calcium Or vitamin D, RECORD): a randomised placebo-controlled trial.* Lancet, 2005. **365**(9471): p. 1621-8.
334. Messerer, M., S.E. Johansson, and A. Wolk, *The validity of questionnaire-based micronutrient intake estimates is increased by including dietary supplement use in Swedish men.* J Nutr, 2004. **134**(7): p. 1800-5.
335. Serra-Majem, L., et al., *Dietary assessment methods for intakes of iron, calcium, selenium, zinc and iodine.* Br J Nutr, 2009. **102 Suppl 1**: p. S38-55.
336. Bolland, M.J. and I.R. Reid, *Calcium supplementation and cancer incidence.* Am J Clin Nutr, 2008. **87**(3): p. 792-3; author reply 793-4.
337. Bonnick, S., et al., *Treatment with alendronate plus calcium, alendronate alone, or calcium alone for postmenopausal low bone mineral density.* Curr Med Res Opin, 2007. **23**(6): p. 1341-9.
338. Peacock, M., et al., *Effect of calcium or 25OH vitamin D3 dietary supplementation on bone loss at the hip in men and women over the age of 60.* J Clin Endocrinol Metab, 2000. **85**(9): p. 3011-9.

339. Smith, E.L., et al., *Calcium supplementation and bone loss in middle-aged women.* Am J Clin Nutr, 1989. **50**(4): p. 833-42.
340. Elders, P.J., et al., *Calcium supplementation reduces vertebral bone loss in perimenopausal women: a controlled trial in 248 women between 46 and 55 years of age.* J Clin Endocrinol Metab, 1991. **73**(3): p. 533-40.
341. Schabas, R., *Artifact in the control group undermines the conclusions of a vitamin D and cancer study.* Am J Clin Nutr, 2008. **87**(3): p. 792; author reply 793-4.
342. Hofstad, B., et al., *Growth and recurrence of colorectal polyps: a double-blind 3-year intervention with calcium and antioxidants.* Digestion, 1998. **59**(2): p. 148-56.
343. Majumdar, S.R., R.H. Fletcher, and A.T. Evans, *How does colorectal cancer present? Symptoms, duration, and clues to location.* Am J Gastroenterol, 1999. **94**(10): p. 3039-45.
344. Lewis, J.R., K. Zhu, and R.L. Prince, *Adverse events from calcium supplementation: relationship to errors in myocardial infarction self-reporting in randomized controlled trials of calcium supplementation.* J Bone Miner Res, 2012. **27**(3): p. 719-22.
345. Sanders, J.L., et al., *Ca(2+)-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells.* Am J Physiol Endocrinol Metab, 2001. **281**(6): p. E1267-74.
346. Ritchie, C.K., et al., *Effects of the calciotrophic peptides calcitonin and parathyroid hormone on prostate cancer growth and chemotaxis.* Prostate, 1997. **30**(3): p. 183-7.
347. Bailey, R.L., et al., *Estimation of total usual calcium and vitamin D intakes in the United States.* J Nutr, 2010. **140**(4): p. 817-22.
348. Reid, I.R., M.J. Bolland, and A. Grey, *Effect of calcium supplementation on hip fractures.* Osteoporos Int, 2008. **19**(8): p. 1119-23.
349. Feskanich, D., et al., *Milk consumption during teenage years and risk of hip fractures in older adults.* JAMA Pediatr, 2014. **168**(1): p. 54-60.
350. Kalkwarf, H.J., J.C. Khoury, and B.P. Lanphear, *Milk intake during childhood and adolescence, adult bone density, and osteoporotic fractures in US women.* Am J Clin Nutr, 2003. **77**(1): p. 257-65.